The Neurological Consequences of Non-Freezing Cold Injury



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ABSTRACT

Non-freezing cold injury is a clinical syndrome characterised by altered sensory perception following prolonged exposure to cool temperatures. Acutely there is loss of sensation but after several hours or days, pain emerges in the distal extremities, which may last months or years after injury. The pain is characteristically more severe when the affected skin is cooled, at temperatures that would not have elicited pain before injury. The condition is well known in the military, most notoriously in The First World War as "Trench Foot". New cases are still seen in the British Military in the 21st Century. There has never been a detailed study to address the neurological phenotype of patients with chronic symptoms. In this thesis 80 such patients underwent neurological phenotyping and assessment for the presence of neuropathic pain. The data in this thesis provide clear evidence for the presence of a sensory neuropathy with severe neuropathic pain due to small fibre neural injury, as demonstrated by abnormalities on quantitative sensory testing and reduced intraepidermal nerve fibre densities. Soldiers of African heritage have long been recognised to be predisposed to injury, raising the question of whether there could be a genetic contribution to this syndrome. Using a whole genome sequencing approach, a variant in the voltage gated sodium channel gene, *SCN9A*, was found to be over-represented in the cohort. Furthermore, in cellular electrophysiology experiments, the variant bestowed gain of function effects on HEK293 cells, but only at cool temperatures, providing a molecular correlate for the cold-aggravated pain seen in patients. Assessment of axonal excitability by threshold tracking suggests that there may be a reduction in slow potassium current, which has been shown to associate with neuropathic pain and cold allodynia. This thesis provides an evidence base for assessment and management of patients and suggests abnormalities in both sodium and potassium channel function, which warrant further research. This could have important implications for targeted therapy.

Acknowledgements

I am very grateful to all the participants who took part in this research, at times in their lives when they were experiencing huge distress and uncertainty about their futures. There would be no data without their commitment. Their contributions will help prevent others going through the same turmoil.

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I would like to thank Dr Iulia Blesneac, who undertook the cellular electrophysiology experiments. She showed great interest in the research and took the time to explain her techniques and to answer my many questions. Dr Carl Fratter performed Sanger sequencing of *SCN9A* and I am very grateful for his vital contribution. I am grateful to all the members of The Neural Injury Group for their collective advice, support, friendship and good humour.

Declaration

I declare that the work contained within this thesis is my own, unless otherwise specified. Notable contributions were made by:

- Dr Andreas Themistocleous performed the staining of skin biopsies and counting of intraepidermal nerve fibre densities for NFCI participants, detailed in Chapter 2

- Dr Carl Fratter performed Sanger Sequencing of SCN9A, as detailed in Chapter 3

- Dr Iulia Blesneac performed the cellular electrophysiology experiments detailed in Chapter 3.

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1 Introduction

1.1 A definition of Non-Freezing Cold Injury

There is no universally agreed upon definition of Non-freezing cold injury. However, it is widely accepted that it is a clinical syndrome comprising skin changes and sensory symptoms, that occurs following (generally) prolonged exposure to cool temperatures insufficient to cause soft tissue freezing (Heil et al., 2016; Imray & Oakley, 2005) The ambient temperature is usually between zero degrees Celsius and 15 degrees Celsius. The absolute temperature, and duration of exposure, required to induce injury varies between patients. Although studies are inconsistent, important additional variables may include environmental conditions (in particular, wetness), as well as the presence or absence of protective clothing, nutritional status, degree of mobility/immobility at the time of exposure and smoking status (Kuht et al., 2019)**.**

The term is used in two contexts: firstly it is an umbrella term that encapsulates the observances made during several military conflicts since the Napoleonic era (including Trench Foot and Immersion Foot, as discussed below), and more pertinently to this thesis, it is used to refer to contemporary cases.

1.2 Historical context of Non-Freezing Cold Injury

The origin of the term Non-Freezing Cold Injury, as defined in the opening to this chapter, is uncertain. It was used as early as 1951 to describe mild cases of "Trench Foot" (Shumacker, 1951)**,** that term coined during the Frist World War. However, it is quite clear that syndromes resembling NFCI, arising from prolonged exposure to cold temperatures, were recognised as far back as the Napoleonic era. Given that much of what we do know of the condition is derived from military experience, it is apposite to frame the history of NFCI with reference to conflicts: Pre First World War, First World War, Second World War and post Second World War (Francis, 1984; Paton, 2002; Whitaker, 2016)

1.2.1 Pre-First World War

Whether or not descriptions of injuries sustained in cold weather conflicts prior to WWI can be said to be truly "non-freezing" is debatable. Certainly, focal syndromes with skin and sensory changes affecting the feet, and following prolonged cold exposure were noted. The temperature at which these were incurred is not entirely certain. At the time no clinical distinction was made between frostbite and NFCI. The first documented descriptions of symptoms potentially compatible with NFCI were written by Napoleon's Surgeon General, Baron Larrey, based on his observations during the Napoleonic campaigns in Russia (1812). He described soldiers with 'vivid pains in the feet, congestion, heaviness and unpleasant creepings in the extremities' (Paton, 2002), all of which occurring at temperatures above freezing, when recent ice had thawed. However, whilst symptom onset seemed to be after thawing, it is possible that injury itself was actually incurred during freezing temperatures. During the first winter of the Crimean War (1853-1856), there were 1942 cases of "frostbite"

reported from a total of 50,000 men at a time when it is known that the temperature was rarely below freezing (Francis, 1984**).** Soldiers would frequently spend 24 hours or more in the trenches, knee deep in mud and snow (Paton, 2002).

Following the Crimean War, thousands of cases were reported during the American Civil War (1861-1865), the Franco-Prussian War (1870-1871, the Russo-Turkish War (1877-1878), the Sino-Japanese War (1895), the Russo-Japanese War (1904-1905), the Boer War (1899-1902) (Paton, 2002) and the Balkan War of 1913 (Smith et al., 1915).

1.2.2 The First World War

The terms "trench foot", "so called" frostbite, "puttee bite" (Johns, 1916) and "trench frostbite" (Smith et al., 1915) were coined during the First World War, to denote the syndrome afflicting tens of thousands of troops on the front line. It was during this period that a distinction was clearly noted between cases of true frostbite, and those of injuries sustained following prolonged exposure to cold temperatures insufficient to cause tissue freezing. Lorrain Smith noted in 1915 that cold injuries were "broadly divisible into two groups ….. the first of which includes the conditions where extreme cold directly and immediately causes death of the tissues, while the second comprises the conditions where cold first of all originates an impairment of the vitality of the tissues with or without subsequent death of the affected cells" (Smith et al., 1915).

There was an epidemic of cases during this conflict, experienced by all sides and on all fronts, as reviewed by Paton in 2002 (Paton, 2002)

It was during the First World War that the first detailed case series were written, clearly documenting the phenotype of the acute phase of the condition. This will be discussed in more detail later, but it is noteworthy that gross tissue destruction and gangrene were frequently prominent features, which is in stark contrast to the contemporary cases, which are the subject of this thesis.

This was also the first time that a clear predisposition to injury of African individuals was observed, Mignon (the French Medecin Inspecteur General**)** documenting that 1,225 cases "frozen feet" were reported among Senegalese troops on the French western front during only two days in mid-April, 1917, at a season when cold injury among French troops was negligible (Paton, 2002). Whether or not this disparity was due to biological differences is unknown. It is quite possible that the Senegalese troops had different, less protective footwear or were subjected to greater exposure than their white counterparts. Nevertheless, this hint of ethnic predisposition is particularly relevant to the contemporary condition and to the participants studied in this thesis.

1.2.3 The Second World War

A similar syndrome was observed during the Second World War, but in this conflict, a novel setting for injury was commonly the ocean rather than the mud of the trenches (although there were massive casualties from trench foot and frostbite), and therefore the term "Immersion Foot" was coined. Thousands of sailors and pilots spent days or weeks in lifeboats, awaiting rescue, their feet submerged in cold sea water. As had been noted by Lorrain Smith in 1915, in 1942 and 1945 Ungley stressed that "the essential cause of immersion foot is prolonged exposure of the limbs to cold insufficient to freeze the tissues"

(Ungley et al., 1945). It was Ungley who delineated different chronological phases of symptomatology for the first time, as will be discussed further in the next section of this introduction. He also made note of the apparent predisposition of individuals of colour: “Coloured races are said to be more prone to trench foot than northern races. In this study, Arabs fared no worse than Europeans” (Ungley, 1942)

It was during and immediately after the Second World War that the first reports of the chronic effects of NFCI were published, (Abramson et al., 1946; Paddock, 1946; Redisch et al., 1951)**,** which will be discussed further in the introduction to Chapter 2. Pain was an important and frequent complaint, and it was specifically exacerbated by exposure to previously innocuous cold environments. This had consequences for the sorts of employment that retired soldiers were able to undertake, presaging what is seen in contemporary cases of NFCI in the British Army.

It was also during this period that civilian cases of a similar syndrome were documented amongst those sheltering from bombs overhead (“Shelter Foot”), although in these cases, the relative importance of cold over limb constriction and immobility is uncertain (Greene, 1941).

1.2.4 Post Second World War

Several conflicts after 1945 have seen cases of frostbite or NFCI, including the Korean War (Orr & Fainer, 1952), The Vietnam War (these were cases of tropical immersion foot or "Paddy Foot", which were sustained in warmer water than other manifestations of NFCI but tends to be categorised amongst these conditions (Akers, 1974; Allen & Taplin, 1973)) and The Falklands Conflict (Golden et al., 2013)**,** again with ethnic predisposition noted (albeit to freezing rather than non-freezing cold injury; at the regimental level cases in black soldiers occurred at a rate of 35.9 per 1000, compared to 5.8 per 1000 in white soldiers (Orr & Fainer, 1952)**)** as well as a chronic pain syndrome (Blair et al., 1957).

However, this is not only a condition of wartime. It is still seen commonly during peacetime military exercises (Burgess & Macfarlane, 2009; Hawryluk, 1977; Kuht et al., 2019; Mitchell et al., 2012; Tek & Mackey, 1993). The condition contributed to, or was solely responsible for, the medical discharge of 518 service personnel from the British army between 2005 and 2014 (Kuht et al., 2019)**.** Reinforcing what had already been observed regarding the heightened risk of African soldiers, Burgess *et al*, in a retrospective case series, calculated a risk of up to 30 times that in white British troops (Burgess & Macfarlane, 2009).

Additionally the syndrome is recognised in the civilian population, as a result of recreation (Fraser & Loftus, 1979), occupation e.g. slaughterhouse workers (Buzanello & Moro, 2012) and social deprivation in the homeless population (Irwin et al., 1997; Olson & Kman, 2015; Wrenn, 1991). Ironically it has even been seen as a result of the attempt to alleviate the pain associated with erythromelalgia (discussed in the genetics section of this introduction), a

condition characterised by neuropathic pain which is exacerbated by warmth and alleviated by cooling (Davis, 2013).

1.3 Clinical phenotype – acute and later stages

The study by Ungley *et al* (Ungley et al., 1945), serves to demonstrate that different phenotypic phases exist from acute to chronic manifestations. This was the first publication to attempt to make such a delineation, which is pertinent to this thesis, which specifically addresses the chronic phase.

1.3.1 Acute Phenotype

Descriptions of early symptoms include visible skin changes such as swelling and discolouration, as well as coldness to touch. In the first one to two hours, the major sensory disturbance is numbness, rather than pain, and there may even be paralysis of movement at the ankles and toes. In his seminal description of some of the first cases of trench foot, Lorrain Smith (Smith et al., 1915) studied 51 cases returning to The United Kingdom from the Front Line. In the acute phase, the men suffered from “numbness and paralysis”, and a frequent description of a sensation “as though walking on stilts” was given or their feet feeling like logs. Patients described a pain on recovery, akin to the “feeling of recovery from sleep”, of their feet. Other cases described a burning pain, worse at night time (Smith et al., 1915).

In a letter to The Lancet in 1915, Dr Porter Parkinson wrote of his observances of 'several' soldiers invalided from The Western Front. Patients reported to him that, acutely, their feet were painful and numb but that there was no anaesthesia to touch, prick, heat or cold. Similarly, to Lorrain Smith, he noted that pain was at its worst at night-time. Interestingly he drew comparison with erythromelalgia (the inherited form of which is discussed later in this chapter), concluding that the condition arose due to damp and cold acting on vasomotor and sensory nerves. Writing in The British Medical Journal in 1917, Dobson recorded his observations, reiterating Lorrain Smith's own account that pain is worse at night, and "neuralgic" in type (Dobson, 1917).

Various macroscopically evident skin manifestations were observed, from discolouration, to "massive peeling and desquamation", through to ulceration, necrosis and gangrene, with a description of affected portions being "black and mummified" (Smith et al., 1915). Some, however, had no such evident skin pathology, and despite sensory symptoms and signs, these were termed "functional" or "hysterical" cases by some authors at the time (Fearnsides and Culpin, 1915)

Ungley was the first author to define different phases of NFCI, which he termed 1) prehyerpaemic, lasting hours to days, 2) hyperaemic, lasting 6-10 weeks, 3) post hyperaemic lasting weeks or months and 4) "late sequels", symptoms ongoing into the second year post injury (Ungley, 1942).

Two publications by Ungley *et al*, published in 1942 and 1945 described a series of up to 80 cases of "Immersion Foot", which he also termed "vasoneuropathy after chilling" (Ungley,

1942; Ungley et al., 1945). Having been shipwrecked, sailors' injuries were sustained during exposure to cold sea water, in rafts or open boats. Exposures lasted up to 14 days. Descriptions of symptoms in the acute phases included "walking as if on cotton wool", cramps, clumsiness tingling and pins and needles. Evocatively, pain was described as "shooting or stabbing, not unlike the lightning pains of tabes dorsalis" (this is relevant as these pains were established to be due to neural injury resulting from syphilis (Hughlings-Jackson, 1880)), and occurring in bursts like machine gun fire, radiating from the centre of the foot outwards, usually to the tips of the toes". These publications include progress charts for individual patients, clearly documenting gross sensory deficits lasting up to 8 months after rescue (Figure 1.1) and Ungley also described "painful over-reaction to light handling or stroking" (i.e. allodynia – Frederick Johns had also written, in 1916, that there were patients in whom "the slightest touch would make them wince (Johns, 1916)).

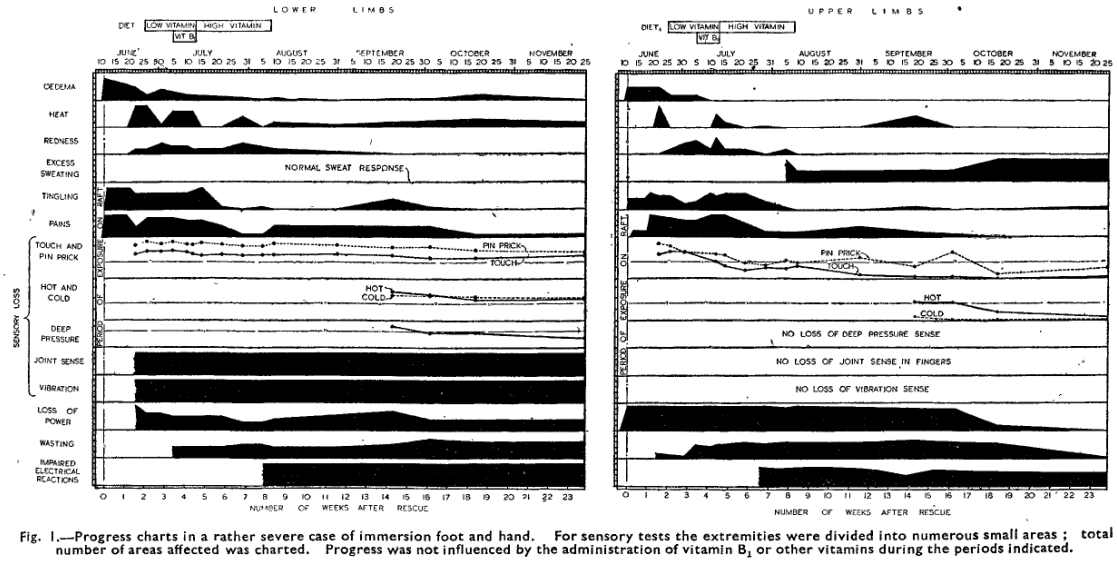


Fig. 1.—Progress charts in a rather severe case of immersion foot and hand. For sensory tests the extremities were divided into numerous small areas; total number of areas affected was charted. Progress was not influenced by the administration of vitamin B_1 or other vitamins during the periods indicated.

Figure 1.1 Clinical assessment chart for one patient, taken from Ungley's 1942 publication (Ungley, 1942). The chart depicts the presence of neurological symptoms and signs in the lower (left panel) and upper (right panel) limbs. Additionally impaired electrical reactions are noted.

Ungley detetmined four grades of severity: those in which there was no neurological dysfunction, those with impairment to light touch and vibration sense at the soles of the feet or distal toes, those with motor weakness in addition to loss of touch and vibration sense, and those with frank gangrene. Similarly, White reported on 15 cases, with pathology, of Immersion Foot, finding sensory abnormaliteis in many cases (Figure 1.2). White noted that

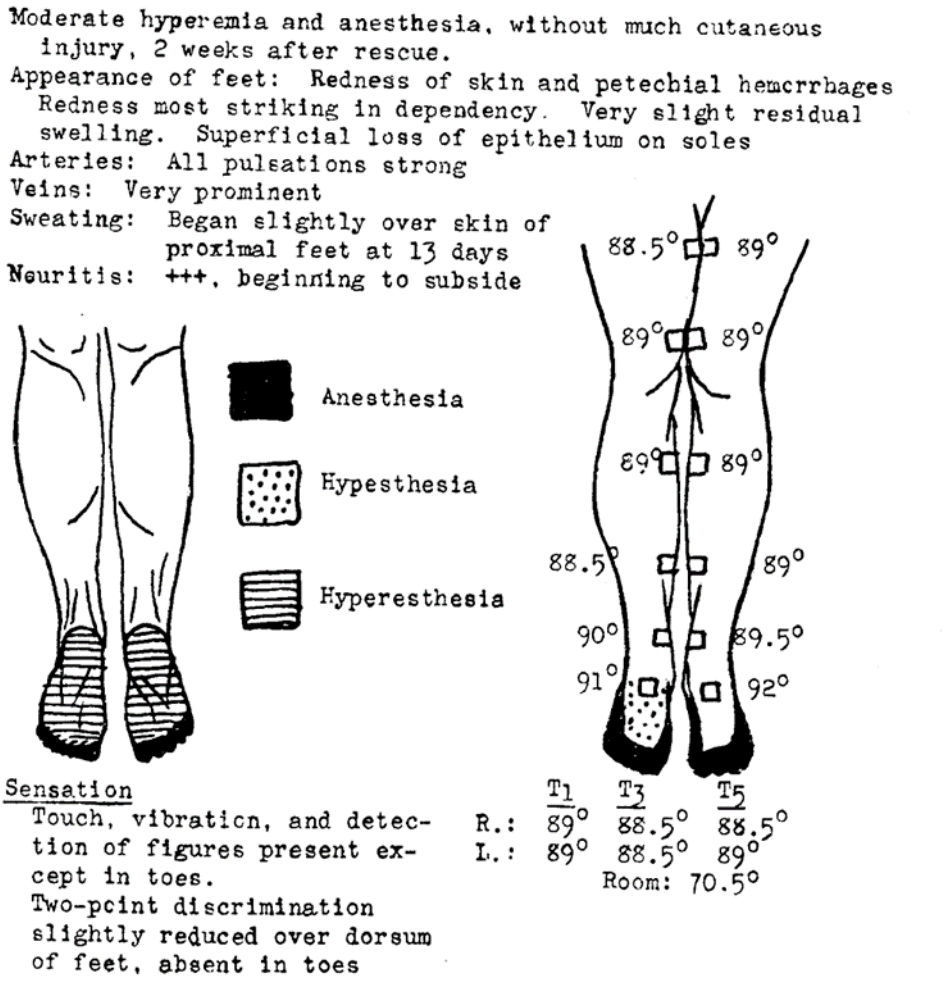


Figure 1.2 Depiction of sensory abnormalities in one case in the hyperaemic phase. From White (White, 1943). Reproduced with permission from The New England Journal of Medicine, Copyright Massachusetts Medical Society

there was an inability of the feet to sweat in these acute cases, observing that this is unsurpring given the similarities between sudomotor and pain fibres in terms of size, degree of myelination and rates of conduction. It was also evident that patients who showed the

most definite signs of nerve and tissue damage were generally the ones that developed the severest degrees of “neuritic pain” (White, 1943).

An important difference between the cases seen in the World Wars, and those seen in The British Army now, is that the acute injury is much more subtle (Kuht et al., 2019). Gangrene is not seen, whereas this was commonplace in the conflicts of the 20th Century, with secondary infection frequently described and amputation relatively common.

1.3.2 Chronic Phase

The proportion of cases whose symptoms do not remit after several months, and who go on to have chronic symptoms, is unknown. Ungley wrote that this was a relatively uncommon occurrence and that pain after several months was rarely encountered, although he did acknowledge that it happened. However it is clear from subsequent series of both late NFCI and frostbite, that many cases do experience pain, frequently described as “burning” or “neuritic” (Abramson et al., 1946; Redisch et al., 1951), as well as tingling and numbness, lasting months or years after initial injury, and with prominent “cold sensitivity” (Blair et al., 1957). The extent of any neurological examination in these studies was highly variable, and little emphasis was placed on sensory examination despite these symptoms being so pronounced. The studies that have investigated patients in the chronic phase will be discussed in the introduction to chapter 2.

This late phase is relevant as it continues to affect soldiers in the British Army, resulting in medical discharge and difficulty obtaining emplyoment in civilian life (Kuht et al., 2019), and it is this population that is under investigation in this thesis.

To summarise this part of the introduction:

1) Non-Freezing Cold Injury has been recognised for more than 100 years
2) Several pieces of evidence in the intervening years point to neurological involvement in the injury caused by exposure to cold temperatures
3) Chronic, disabling pain is observed as a feature in the late stages of the condition. This pain is characteristically elicited or exacerbated by exposure to cool temperatures that would not have elicited pain prior to injury
4) Acutely, non-freezing cold injury presents very differently in the 21st Century from its descriptions in the first half of the 20th Century, with a much subtler phenotype
5) It was evident even 100 years ago that there might be an ethnic predisposition to this condition, which was reiterated later in the 20th century, and is now clearly established to be the case.

1.4 The structural components of the peripheral sensory somatic nervous system

In the next section the neural pathology associated with NFCI will be discussed. It is clearly impossible to perceive any sensory stimulus without the central nervous system, the many neural pathways within the spinal cord and brain performing incredible integration and

modulation of stimuli arriving from the periphery. This thesis is concerned with the peripheral somatosensory nervous system, however, and the published reports of neural pathology in NFCI are focussed almost exclusivelty on the periphery.

The peripheral sensory system comprises differently sized neurons whose cell bodies are located within dorsal root ganglia (DRG) and trigeminal ganglia, from where they send a peripheral axon to their target (skin, muscule, joints, cornea) and a central projection to the superficial dorsal horn of the spinal cord (they are pseudounipolar). Here they synapse onto second order neurons that ascend the spinal cord in dedicated tracts depending on subtype.

Sensory neurons are generally classified according to their size (small or large) and degree of myelination (unmyelinated, thinly myelinated or thickly myelinated), with fibres of different sizes conveying broadly different sensory modalities. Sensory fibres can also be classified according to their thresholds for activation (whether high or low threshold stimulus intensity is required for activation).

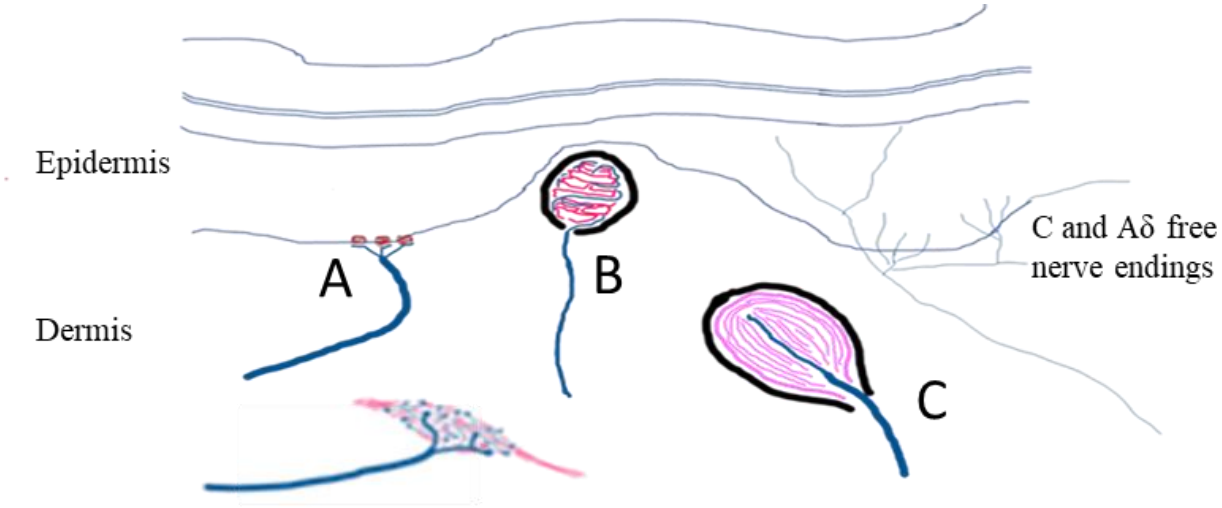
In order of decreasing size the major classes of sensory neurons are Aβ fibres, Aδ fibres and C fibres. The Aβ fibres are thickly myelinated and rapidly conducting, conveying predominantly innocuous touch and proprioceptive input. In the skin these low threshold mechanoreceptors innervate specialised mechanosensory end organs, particularly in glabrous (non-hairy) skin (Figure 1.3), as well as hairs, disciminating between such innocuous sensations as indentation, vibration or stretch and whether these stimuli are static or dynamic (Roudaut et al., 2012; Zimmerman et al., 2014). There is growing evidence for

an additioanl role of some Aβ fibres in pain perception (Nagi et al., 2019), and certainly an established role in pain modulation (Jensen & Finnerup, 2014).

Aδ and C fibres are thinly myelinated and unmyelinated, respectively. These comprise mechanical and thermal nociceptors, thermoceptors and also low threshold mechanoreceptors (Aδ-LTMRs, also known as D-haris – their existence in humans, unproven, - and C-LTMRs). As with low threshold Aβ fibres, these are associated with differing types of hair follicle (Li et al., 2011) and like some Aβ fibres, C-LTMRs may also modulate pain (Habig et al., 2017)

There are varying degrees of polymodality such that different subpopulations of Aδ or C fibres may respond to more than one type of stimulus (Table 1.1) (Ackerley & Watkins, 2018; Lawson et al., 2019)**.** Thermal and nociceptive neurons have their free endings in the dermis and epidermis, the latter of which may be quantified in research and clinical practice by analysis of skin biopsy specimens (Lauria & Devigili, 2007).

There is considerable molecular heterogeneity between different neuronal subpopulations, bestowing them with varying degrees of specificity with respect to the modalities they convey (innocuous versus noxious thermal and mechanical stimluli). The molecular phenotype of these neurons is not static, such that in pathological states, there may be increases or decreases in the expression of particular molecules.



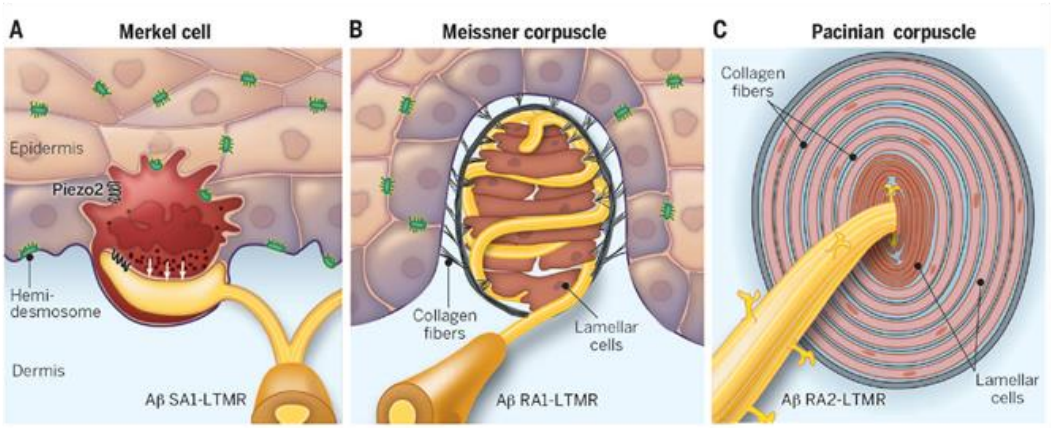


Figure 1.3 Low threshold mechanoreceptors (LTMRs). Fibres innervate different types of hair follicle and specialised somatosensory end organs (figure taken from Zimmerman et al., 2014 - From The gentle touch receptors of mammalian skin *Science* 21 Nov 2014: Vol. 346, Issue 6212, pp. 950-954 DOI: 10.1126/science.1254229. Reprinted with permission from AAAS)

SA – Slowly adapting, RA – Rapidly adapting, LTMR – Low threshold mechanoreceptor

Fibre type	Subtype	Further subtype	Stimulus sensitivity
C			
	C-mechanosensitive (CM) nociceptor, type 1A, polymodal	C-mechano-heat nociceptors (CMH)	Noxious touch; subclasses also respond to temperature
		C-mechano-heat-cold nociceptors (CMHC)	
	C-mechanoinsensitive (CMi) nociceptors, type 1B	C-mechanoinsensitive-heat-insensitive nociceptors (CMiHi)	Noxious heat, little mechanical sensitivity. CMiHi have little thermal sensitivity
		C-mechanoinsensitive histamine positive [CMi(His+)], C-pruritic, C-itch	Pruriceptors sensitive to histamine
	C-cold, type 2	No defined subpopulations	Cooling, with no sensitivity to touch. Activity at typical skin temperature and fire down to 0°C
	C-warm	Low-threshold warm receptors	Warming, no sensitivity to touch
		High-threshold warm receptors	
	C-low-threshold mechanoreceptor (C-LTMR)	No defined subpopulations	Preferential response to slow, gentle touch, at room temperatures
Aδ	Low threshold cold-sensitive	No defined subpopulations	Innocuous cold
	Aδ-mechanosensitive, polymodal	Aδ-mechano-heat-cold	Noxious mechanical, heat and cold
		Aδ-mechano-cold	Noxious mechanical and cold
	Aδ-mechanoinsensitive cold-sensitive		Innocuous or noxious cold
	Aδ-low threshold mechanoreceptors (also known as D-hairs)		Gentle touch of hairy skin

Table 1.1 There are several subtypes of Aδ and C fibres, with varying degrees of stimulus specificity (Existence of D-hairs in humans, unproven)

1.5 Pathological evidence for neurological involvement in NFCI

1.5.1 Observations in animals and humans

As noted by Nukada as late as 1981, "despite the plethora of clinical material and a number of experimental studies the morphological accompaniments of non-freezing injury to nerve remain uncertain" (Nukada et al., 1981). This remained the case up until the time the work in this thesis was commenced, particularly for cases in which injury was sustained more than one year previously. Both before and after Nukada's statement, the available pathological evidence is conflicting. Some studies found preferential small fibre involvement, whilst others found exclusively large fibre involvement. This may in part be due to the use of different experimental paradigms and organisms used as animal models. In the case of the human cases studied, the severity of injury was variable. In stark contrast to present day NFCI cases, gangrene and gross tissue destruction were frequent findings. In addition the histological techniques available were not as advanced as those at modern day disposal (immunohistochemistry, especially) Despite the fact that intraepidermal nerve fibres had been described as early as the mid-19th Century (Langerhans, 1868), their existence was debated, and they were not systematically examined until as late as 1959 (Abels, 2014; Arthur & Shelley, 1959; Lauria, 1999).

Friedman examined pathological specimens from 14 soldiers who sustained NFCIs at The Battle of Attu, in 1945 (Friedman, 1945). Confounding factors include the presence, in all cases, of haemorrhage, bullae, infection or gangrene of the foot. In early cases (specimens obtained from soldiers who had died 7-10 days post cold exposure), abnormalities were

found only in large, myelinated nerve fibres. These were frequently “broken, beaded and frayed”, and abnormalities were observed in axis cylinders as well as in myelin. Demyelination was prominent in late cases, and there was “disappearance of axis cylinders” as well as oedema and perineural fibrosis. Small myelinated and unmyelinated fibres, however, had normal appearances.

Blackwood described the pathological findings from 14 cases of immersion foot during WWII (Blackwood, 1944). The majority of these were sustained following days of exposure in open top boats at sea. One case resulted from days of lying in an unheated pill box on a Scottish hillside. Specimens were obtained from patients who had died shortly after exposure, due to associated illness, as well as from those who had undergone amputation up to 12 months following injury. Specimens included entire feet (or hands), as well as whole digits and whole tibial nerves. A range of pathology was seen from loss of corpuscles in the skin, through to extensive Wallerian degeneration. Both motor and sensory nerves were affected.

As well as the observations made in humans, many attempts have been made to create animal models, with a variety of different species and methodologies used **(**Table 1.2). As might be expected from such varied approaches, the findings of these studies were also variable, with some documenting predominantly large fibre injury, and others small fibres. Several of the experiments failed to recreate the circumstances in which human casualties came to be injured. For example, in several instances large nerves were dissected out and exposed directly to a cold environment, such as experiments in which the sciatic nerve was placed in a cold cuff or a cold probe applied directly to it. These approaches could only be said to be

useful for assessing the effect of cold on an exposed sciatic nerve, and even then, only in the species being used. Another approach bears more resemblance to the human condition, in that the distal portions of intact limbs were exposed to cold (with or without wetness) environments for prolonged periods prior to sacrifice.

Author, Date	Human/Animal context	Pathology
1942 (Ungley, 1942)	Biopsies of interdigital nerves of feet affected by sepsis and gangrene. Amputated lower limbs (4- and 11-months post injury)	From biopsies: Complete Wallerian degeneration of interdigital nerves without vascular change From amputated limbs: Severe degeneration of main nerve trunks and early regeneration Small fibres not studied
1944 (Blackwood, 1944)	Pathology specimens from 14 severe cases of Immersion Foot ranging from digital nerve to whole leg. Time from injury to specimen removal ranged up to 26 months.	Loss of myelinated nerve fibres. Schwann cell proliferation. Regenerating nerve fibres. Cutaneous fibres studied in two of the cases, one who died during exposure, and another 12 months after exposure. Cutaneous fibres showed no abnormality in the former. In the latter there were no visible Meissner's Corpuscles in the toes. Pacinian Corpuscles seen but un-innervated.

1945 (Friedman, 1945)	Fourteen cases, including some post mortem	Axonal degeneration and demyelination preferentially affecting large nerves. Normal small and unmyelinated fibres.
1915 (Smith et al., 1915)	Rabbits' feet exposed to cool, damp mud for varying durations	Oedematous swelling of axis cylinders but no degeneration
1943 (Blackwood, 1943)	Rats exposed to temperatures of 3-5°C between 12-96 hours. Tails examined postmortem	No significant abnormalities in rats exposed for 24 hours. Progressively more severe Wallerian degeneration of myelinated fibres at exposures of 48 hours or longer, increasingly pronounced the longer after exposure that tails were examined. No study of small fibres mentioned
1944 (Large & Heinbecker, 1944)	Dogs' forelimbs immersed in water at 6°C for 96 hours. Ulnar nerves studied	Demyelination and degeneration of myelinated and small, unmyelinated fibres
1945 (Denny-Brown et al., 1945)	Direct cooling (8°C) of cat sciatic nerves for as little as 30 minutes	Selective loss of large, myelinated fibres. No abnormality of small fibres. Sympathetic fibres were the most resistant of all. Not possible to say whether axonal degeneration or loss of myelin is the primary process. There was complete regeneration of nerve fibres and myelin at 3 months

1953 (Pirozynski & Webster, 1953)	Rabbit ears exposed to temperatures of -20°C to -30°C for less than 5 minutes	Progressive degeneration of all fibre types, generally thinly myelinated or unmyelinated first, followed by larger fibres. At two hours post injury, swelling at Nodes of Ranvier in thickly myelinated fibres and marked swelling of small fibres. At 12 hours post injury greater swelling of large fibres with elongated Nodes and frank disintegration of smaller fibres. At 72 hours post exposure, no morphologically intact fibres. Smaller fibres degenerated and disintegrated before large fibres.
1955 (Douglas & Malcolm, 1955)	Cat saphenous and sciatic nerves	Blockade of firing affecting thinly myelinated>thickly myelinated>small unmyelinated fibres
1967 (Schaumburg et al., 1967)	Animal – cats. Cold probe inserted for various durations under sciatic nerve	Cold dose dependent impairment in motor>sensory function. Conduction block. Non preferential (size) nerve fibre degeneration
1973 (Basbaum, 1973)	Animal – cats.	Wallerian degeneration and segmental demyelination. Unmyelinated fibres unaffected
1981 (Nukada et al., 1981)	Animal – rat model. Sciatic nerve subjected to cooling *in vivo*	Light and electron microscopy showed demyelination and a delayed axonal degeneration

1991 (Kennett & Gilliatt, 1991b)	Rabbits. Local cooling of tibial nerve *in vivo* to 5°C and 1-2°C for 2 hours	Earliest change was axon swelling of myelinated fibres with some loss of myelin but no change in unmyelinated fibres. Later Wallerian degeneration of distal segments, lesser loss of unmyelinated axons. Preferential loss of large fibres
1991 (Kennett & Gilliatt, 1991a)	Rabbit hindlimb immersed in 1-2°C for up to 14 hours	Earliest change was swelling of myelinated fibres and discontinuity of myelin with no change to unmyelinated fibres. Three weeks post immersion the greatest reductions in number were for large fibres. Greater injury proximally than distally.
1996 (Irwin, 1996)	Rabbit hindlimb immersed in 1-2°C water for 16 hours	Preferential involvement of large, proximal myelinated fibres with distal extension as duration of exposure increased. Reduction in mean axon diameter in mixed nerves of animals subjected to longer periods of immersion. Significantly less subepidermal nerve fibre staining in immersed limbs compared to controls.
1998 (Jia et al., 1998)	Rat sciatic nerves cooled to 3.5°C for 60, 90, 130 or 150 minutes	Structural injury to unmyelinated fibres occurred only after 150 minutes of exposure. Myelinated fibres were selectively affected at durations of 90 minutes or longer

2015 (Li et al., 2015)	Rat Sciatic nerve subjected to cooling *in vivo*	Degeneration of myelinated>unmyelinated fibres. Leakage of blood-nerve barrier in perineurium

Table 1.2 A summary of studies of pathology in cold injury in humans (greyed boxes) and animals

One small study has examined the effect of a procedure known as cryolipolysis or 'coolsculpting', upon human cutaneous nerves (Garibyan et al., 2015). This is marketed as a non-surgical alternative to liposuction, killing adipocytes through application of cold plates to the region of excess adiposity. A well-known side effect of the procedure is a prolonged period of anaesthesia at the site it is performed. Using immunohistochemical techniques not available to investigators in the early and mid-20th Century, the authors of this study showed a marked reduction in unmyelinated intraepidermal nerve fibre density as well as myelinated cutaneous fibres.

Whilst none of the animal studies is ideal in relation to the human condition, it is clear from each of them that cooling of nerve fibres, by whatever means, is associated with neural injury. It is difficult to interpret the pathological data from the studies using tissue from humans, as in these cases injury was frequently complicated by gross destruction of the soft tissues and secondary infection. Nevertheless, taken together it does seem clear that exposure to cold may induce neural injury. The mechanisms by which this might occur are unknown, and relatively little experimental work has been undertaken to answer this question.

1.5.2 Potential mechanisms of cooling induced neural injury

This mechanisms by which cooling causes nerve injury are unknown. Interesting hypotheses and arguments for and against these have been discussed (Jia et al., 1998). Potential mechanisms include 1) ischaemia/hypoxia (Jia & Pollock, 1997), 2) interrupted axonal transport of macromolecules and organelles (Chen et al., 2016; Cosens et al., 1976; Tsukita & Ishikawa, 1980), 3) the accumulation of reactive oxygen species (Geng et al., 2015; Wu et al., 2009) or 4) An excitotoxic mechanism through excessive activation of cold sensing molecules (So et al., 2016).

1.6 Neuropathic Pain – a definition, relevance to NFCI, and how it is studied

1.6.1 A definition

The International Association for the Study of Pain defines neuropathic pain as that which arises as a direct consequence of a lesion or disease affecting the somatosensory nervous system**.** As described above, the somatosensory nervous system comprises all structures in the pathway from primary sensory cortex most proximally to sensory nerve termini most distally, via brain and brainstem structures, the spinal cord, nerve roots, dorsal root ganglia, peripheral nerves, their respective specialised endings and possibly some skin cells.

1.6.2 Criteria for diagnosis of neuropathic pain

The latest iteration of criteria required for diagnosis were published by the Neuropathic Pain Special Interest Group (NeuPSIG) of the International Association for the Study of Pain (IASP) in 2016 (Finnerup et al., 2016)**.** These form the basis of the study design undertaken in Chapter 2. Briefly, the guidelines require that (1) there has been a relevant insult, (2) pain be distributed in a neuroanatomically plausible distribution, (3) that there are sensory abnormalities on examination in the same neuroanatomically plausible distribution, and (4) that a diagnostic tests confirms the presence of a neurological lesion.

1.6.3 Historical clinical and pathological data support the existence of neuropathic pain in NFCI

Investigators reporting cases of acute and chronic cases went as far as to report the pain as "neuritic" (Abramson et al., 1946; White, 1943)**,** although this term was used in a colloquial sense rather than as a result of any diagnostic pathway. When reviewed collectively the published literature does satisfy IASP NeuPSIG diagnostic guidelines for neuropathic pain. There is marked variability in the severity of cases, however, and many of them had considerably more severe injury that is seen today. Furthermore, none of these studies was concerned with the study of pain, specifically, and in many cases if there was an absence of gross tissue destruction, such patients were viewed with scepticism. The historical evidence, with particular emphasis on chronic cases, will be discussed further in the introduction section of chapter 2.

1.6.4 Additional features of neuropathic pain, not required by the guidelines, are also present

Neuropathic pain often has certain characteristics that differentiate it from other sources, including the presence of allodynia (pain evoked by usually innocuous stimuli – as a point of interest perhaps the first modern day description of allodynia was in soldiers injured during The American Civil War**,** as documented by Silas Mitchel Weir in 1872 and republished in 1970 (Mitchell, 1970)) and the qualities of the pain itself (burning, squeezing, electric shocks) and associated sensory symptoms (tingling or pins and needles) (Bouhassira & Attal, 2011; Sun et al., 2018). As already discussed, such descriptors are given by sufferers of non-freezing cold injury in the acute and chronic phases, supporting the idea that their pain may be neuropathic in nature. Furthermore nocturnal worsening of pain as described by some contemporaneous investigators (Dobson, 1917; Smith et al., 1915) is a characteristic feature of neuropathic pain (Burish et al., 2019; Galer et al., 2000)

1.6.5 The study of human neuropathic pain phenotypes *in vivo*

In relation to pain phenotypes there are many different questions a study may seek to answer: Is the pain neuropathic? Are there factors predictive for more severe pain, or for treatment refractoriness? Is there associated psychosocial morbidity? Are there differences in phenotype that might distinguish between different causes of pain? The techniques described below, several of which are used in chapter 2, have been applied to these questions. Importantly they are also applicable to two of the main questions in this thesis: Is the pain experienced neuropathic in nature and, if so, what is the associated somatosensory phenotype?

1.6.5.1 **Questionnaires**

Regardless of aetiology, validated questionnaires can be used to establish specific symptom qualities, as well as their relationships to parameters such as functional impact and psychosocial elements (e.g. presence of anxiety, depression, fear), as reviewed by Attal and Bouhassira (Attal et al., 2018). Some may have an examination component, such as the DN4 questionnaire, which is used for screening purposes. Such questionnaires may be designed to assess for the presence and impact of neuropathic pain in general (Bennett et al., 2007). They may also be used to investigate specific disease states with a neuropathic component, such as back pain (Freynhagen et al., 2006), diabetes, chemotherapy induced peripheral neuropathy (Kanda et al., 2019) carpal tunnel syndrome (Levine et al., 1993) and even very rare diseases such as Fabry Disease (Üçeyler et al., 2014).

1.6.5.2 **Investigations**

Targeted investigations may be used to investigate principally the peripheral nervous system component, (such as nerve conduction studies, microneurography and determination of intraepidermal nerve fibre density), or the central component (principally functional magnetic resonance imaging (Jaggi & Singh, 2011; Moisset & Bouhassira, 2007)). Psychophysical testing (quantitative sensory testing) assesses the whole somatosensory system, but through the interpretation of defined protocols, the relative contributions of different fibre types may be estimated (Baron et al., 2017).

1.6.5.2.1 Nerve conduction studies

Nerve conduction studies are used to assess large sensory and motor fibres in the peripheral nervous system. Whilst these structures do have a role in the modulation of neuropathic pain signals in the periphery, it is the small diameter myelinated fibres and unmyelinated fibres that are the principal mediators of noxious sensation, whether that be appropriate nociception or maladaptive neuropathic pain. Conventional nerve conduction studies are insufficiently sensitive to assess these fibre types but remain useful to assess for co-existing large fibre neuropathy, in which case they may be useful in terms of helping establish aetiology, but also in term of localisation of a lesion.

1.6.5.2.2 Microneurography

Microneurography is a technique which can assess individual nerve fibres of all types, in the periphery. A needle electrode is inserted into the nerve fibre (or nerve fibre bundle) of interest, percutaneously, permitting recording to be made in the awake patient, who is able to provide perceptual information (Ackerley & Watkins, 2018). By application of different mechanical, thermal and chemical stimuli to the skin, it is possible to distinguish different subpopulations within a given fibre type, including C fibres. In a condition such as chronic NFCI this would be advantageous as it would allow measurement of responses to cool and cold stimuli applied to the skin directly, or through manipulation of the ambient temperature of the testing environment. There is precedent for this in the study of experimental human pain (Samour et al., 2015) as well as in a disease state (Serra et al., 2009). Significant disadvantages of this technique are that it takes up to several hours to perform a detailed study and requires a highly experienced technician to identify the nerve(s) of interest, and then to be able to interpret the recording. These factors render this a difficult technique to use, especially in a large cohort of participants.

1.6.5.2.3 Corneal confocal microscopy

Corneal confocal microscopy (CCM) is a non-invasive technique allowing the assessment of density and morphological characteristics of small nerve fibres in the cornea. Commonly used measures are nerve fibre density, nerve fibre length, nerve branch density and tortuosity. With regard to neuropathy, its first use was in the assessment of diabetic neuropathy (Malik et al., 2003; Tavakoli et al., 2010). In the intervening 17 years there has been considerable interest in its application to many other neurological conditions of various aetiologies, including those which are predominantly central disorders, such as Parkinson's Disease, multiple sclerosis and stroke (Petropoulos et al., 2020). Significant differences in CCM parameters have been noted between controls and patients with a variety of neuropathies, as well as between patients with painful versus non-painful neuropathy. Examples include diabetic neuropathy (Kalteniece et al., 2020), HIV associated neuropathy (HIVAN) (Kemp et al., 2017), Charcot Marie Tooth 1A (Tavakoli et al., 2012) and idiopathic small fibre neuropathy (Tavakoli et al., 2010). This technique has not been trialled to assess for the presence of neuropathy in NFCI.

1.6.5.2.4 Quantitative Sensory Testing (QST)

This is a well-established psychophysical technique for the interrogation of the somatosensory nervous system in patients with neuropathic pain (Backonja et al., 2013).

The comprehensive protocol devised by the German Neuropathic Pain Network (Rolke et al., 2006) assesses perceptual thresholds to graded innocuous mechanical and thermal stimuli, noxious thermal and mechanical stimuli, vibration, thermal sensory limen, wind up, and dynamic mechanical allodynia. Absolute values are Z-transformed and results are

compared to control data (Magerl et al., 2010). Values falling outside 2 standard deviations of the control mean represent gain or loss of function for that modality.

As a stand-alone investigation QST it is not specific for localisation of a lesion or disease, nor for aetiology. However, as a complementary test the technique can provide supporting evidence for involvement of particular fibre types and pathophysiological mechanisms in the peripheral nervous system. In addition, abnormalities in some parameters are particularly suggestive of central sensitisation, such as mechanical hyperalgesia and gain of function in wind up ratio.

Principal component and cluster analyses have revealed distinct profiles that associate with particular pain descriptors within aetiologies. Variability within aetiologies is probably as large as between aetiologies (Freeman et al., 2014). Different patterns of somatosensory profile (sensory loss vs thermal hyperalgesia vs mechanical hyperalgesia) may differentiate different pathophysiological mechanisms of pain, and may indicate treatment responsiveness (Baron et al., 2017). Indeed this has been shown to be the case in the responsiveness to oxcarbazepine for patients with painful neuropathy of different aetiologies (Demant et al., 2014).

1.6.5.2.5 Intraepidermal nerve fibre density (IENFD)

The quantification of small fibre innervation of the skin is in widespread use as an objective determinant of small fibre pathology, in research and clinical practice (Lauria & Devigili, 2007). The number of nerve fibres seen to cross from the dermis into the epidermis is counted in skin biopsy specimens and expressed as number of fibres per millimetre of skin. The value obtained is compared to published reference data from age and sex -matched (at present there are no ethnicity specific reference data), healthy individuals. A reduced IENFD satisfies the NeuPSIG criteria as a diagnostic test confirming the presence of a lesion or disease of the somatosensory nervous system (Finnerup et al., 2016). Whilst determination of fibre density is the major clinical measure used, it is possible to undertake analysis of nerve fibre morphology, including the presence of swellings, as well as branch patterns. Skin biopsy also allows imaging of small myelinated fibres, sweat glands, hair follicles and vessels, including arteriovenous anastomoses. These structures are present in different proportions in hairy versus glabrous skin.

1.6.5.2.6 Threshold Tracking

Threshold tracking is a semi-automated technique that allows dynamic study of nerve excitability *in vivo*, providing measures of membrane conductance, and allowing indirect assessment of ion channel function, drawing on established and emerging knowledge of the spatial and temporal distribution of channel activity in axons. This differs from conventional, and widely used nerve conduction studies, which in most cases assess only the proportion of nerve fibres conducting, and the velocity at which they do so. Although useful inferences can be made regarding the type of underlying pathology from these studies, they provide

little insight about the properties of the axonal membrane at the site of stimulation. Although threshold tracking was first described 50 years ago, and has been developed and refined considerably since then, it is still not in routine clinical use, but has been utilised extensively in the research setting for the study of central and peripheral neurological disorders (Bostock et al., 1998; Kiernan et al., 2020). The technique is discussed in detail in Chapter 4.

1.7 Molecular genetics of human neuropathic pain

The human genome (in health) comprises 3 billion base pairs, of which 99.5% are identical between any two humans, with variance limited to between 0.1% and 0.4% (Karki et al., 2015). Variance is brought about through alteration in DNA sequence, which may be inherited from the parents' germlines, or occur *de novo* at any point during development or postnatally. The estimated heritability of pain phenotypes varies between 25% and 50% (Nielsen et al., 2012).

Historically genetic variance has been referred to in different terms, mostly according to their prevalence in the population, where mutations were defined as occurring in less than 1% and polymorphisms in over 1%. This figure of 1% was arbitrarily chosen (Schildgen & Schildgen, 2013). Mutations and polymorphisms have also been distinguished based on disease causing capacity, mutations being disease causing and polymorphisms being benign and non-consequential. However it has become increasingly clear that relatively common single nucleotide polymorphisms can, and do, associate with, cause or predispose to disease states (Karki et al., 2015).

A mutation is a rare event, occurring at a frequency of <1%, that may or may not result in change in phenotype or disease. Different types of mutation include 1) point mutations, where a single nucleotide is added, deleted or substituted, 2) alterations in chromosome structure, such as deletions, duplications or translocations and 3) copy number variation,

Our understanding of the processing of neuropathic pain has been advanced hugely through the study of rare, monogenic conditions of aberrant nociception. The identification of mutated genes in these conditions has enabled targeted research in much commoner human conditions, as well as animal models and *in vitro* systems.

The evidence that particular gene mutations are pathogenic is sometimes clear**,** for example in the cases of rare monogenic inherited phenotypes such as inherited erythromelalgia or paroxysmal extreme pain disorder (as discussed below). In these cases, where a rare mutation is found in kindreds with a clearly identifiable phenotype, and which segregates exclusively with that phenotype, it is relatively straightforward to ascribe pathogenicity to a given mutation.

1.7.1 Rare neuropathic pain disorders exemplify the vital function of voltage gated sodium channels in human nociception

The most eloquent and relevant evidence for the genetic contribution to human neuropathic pain phenotypes derives from study of rare monogenic disorders, which display Mendelian inheritance. In these diseases rare mutations have a large effect size. Dominant and

recessive, gain and loss of function mutations exist. Resulting syndromes include those characterised by excessive pain (e.g. primary erythromelalgia, certain hereditary sensory neuropathies) and those where absence of pain is the cardinal feature, the syndromes of congenital insensitivity to pain (CIPs).

1.7.1.1 **Inherited erythromelalgia**

In the case of erythromelalgia, the clinical syndrome was well recognised long before any causative gene was identified (Mitchell, 1878). Mitchell described eleven cases with variable age at onset. The pain was described as that of burning, mustard or severe sunburn. It was clearly exacerbated by heat, even from bedclothes, and arrested by cold. In addition to the presence of pain, colour changes of the extremities were a striking feature of these cases e.g. *"in the erect position….the foot gets redder and redder….as if a ligature has been tied around the limb".* Another noteworthy feature documented by Mitchell is that the condition was rarely amenable to treatment, and that although cooling was beneficial, the condition could have a propensity to become worse and refractory to cooling over a period of years. Interestingly, Mitchell gives a very brief description of the case of a *"young man, desirous of "hardening" himself, stood in cold water up to his knees for eight mornings. Thereafter walking caused his feet to become cold, white and numb, with aching when walking ceased*". No further information is provided but it is tempting to speculate that this individual was more likely to have suffered NFCI than to have erythromelalgia. Iatrogenic NFCI as a result of trying to alleviate the pain of erythromelalgia has been documented much more recently (Davis, 2013)

In 2004, the causative genetic mutation in inherited cases was identified by linkage analysis, in *SCN9A*, encoding the VGSC, $Na_v1.7$, in two Chinese families (Yang et al., 2004). Since then no fewer than 30 gain of function mutations have been identified in this gene in families affected by the condition (Bennett et al., 2019), as well as in sporadic cases (Han et al., 2006), and in many cases the associated effects on channel function have been characterised, electrophysiologically. Figure 1.5 depicts the known amino acid changes in $Na_v1.7$, which result in gain of function through enhanced activation of the channel.

1.7.1.2 **Paroxysmal extreme pain disorder**

Paroxysmal extreme pain disorder (PEPD) had been recognised as a clinical entity since its first published description in a 9 month old boy, his father and many members of his father's family in 1959 (Hayden & Grossman, 1959). At the time it was termed "an unusual hereditary idiopathic" disorder by the authors. The phenotype in this family was characterised by episodic, very severe rectal pain triggered by defaecation, accompanied by skin colour changes in the buttocks and extremities. As suggested by the diagram of the pedigree (Figure 1.4), this is an autosomal dominantly inherited disorder, but far from being idiopathic, the identity of the causal gene is well established as *SCN9A* (Fertleman et al., 2006) and at least eleven different pathogenic, gain of function mutations have been described (Bennett et al., 2019). Not only have these mutations been identified, but their functional effects on neuronal excitability (an enhancement through gain of function) have been well described. Through different mechanisms, including impairment of channel inactivation, enhancement of recovery from inactivation and the generation of resurgent currents, the likelihood of nociceptive firing is increased, giving rise to pain (Emery et al., 2015).

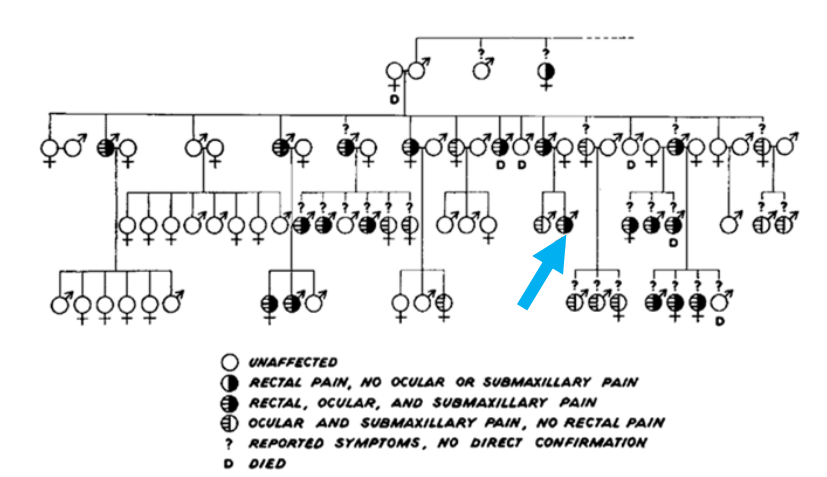


Figure 1.4 Genetic diagram of the family with paroxysmal extreme pain disorder. Adapted from Hayden & Grossman, 1959.

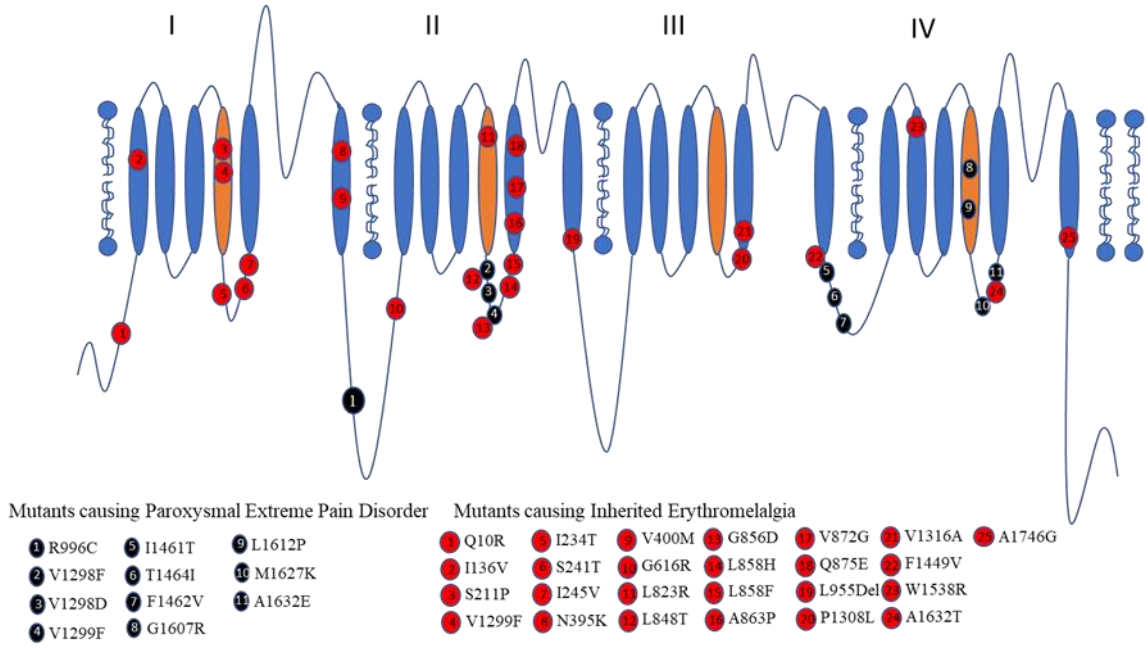


Figure 1.5 $Na_v1.7$ alpha subunit structure showing the locations of amino acids affected by gain of function mutations causing PEPD and IEM. Transmembrane segments are represented by elongated ovals, with orange ovals representing the S4 voltage sensing segment

1.7.1.3 Congenital insensitivity to pain

In stark contrast to PEPD, Congenital Insensitivity to Pain (CIP) is characterised by the hallmark feature of a severely impaired or absent ability to perceive usually noxious stimuli (be they thermal, chemical or mechanical) as painful. Other sensory modalities are (generally) in tact. Again, the phenotype was described long before any genetic cause was

identified (Swanson, 1963; Van Ness Dearborn, 1932). Dearborn's description concerned a single individual, dubbed "The human pincushion" for his ability to withstand usually noxious mechanical stimuli. Swanson's description was of two male siblings. This inability to respond to threatening stimuli renders them frankly harmful, such that tissue damage due to burns, fractures or self-mutilation leads to profound morbidity (Drissi et al., 2020; Thrush, 1973).

The first identified causative mutations for this form of CIP were found in *SCN9A* in 2006 (Cox et al., 2006). These mutations were bi-allelic and caused loss of function. Since then, many additional mutations have been identified in this gene (Bennett et al., 2019), as well as in *SCN11A*, encoding $Na_v1.9$ (Leipold et al., 2013; Woods et al., 2015).

There are mutations in non-sodium channel genes that also result in CIP, but in these cases via a failure of nociceptors to develop at all. Examples include the receptor for Nerve Growth Factor (NGF), Tropomysin Receptor Kinase A (TrkA) (Indo et al., 1996) and PRDM12 (Chen et al., 2015), reviewed in (Nahorski et al., 2015; Rotthier et al., 2012).

1.7.1.4 Rare sodium channel variants are increasingly recognised in commoner neuropathic pain states

Small fibre neuropathy is a common condition, where frequently no underlying cause is identified. Known causes of small fibre neuropathy include diabetes, alcohol excess, HIV infection, chemotherapy and other drugs. There are rarer acquired (e.g. amyloidosis) and genetic (e.g. Fabry's Disease) causes. Cases in which the cause remains unknown are termed idiopathic. Using stringent diagnostic criteria for idiopathic small fibre neuropathy, 8/28 patients with a diagnosis of idiopathic small fibre neuropathy were found to harbour variants in *SCN9A* (Faber et al., 2012). Importantly the specific variants identified rendered dorsal horn neurons hyperexcitable, strongly suggesting that these variants are indeed pathogenic (Han et al., 2012). The same approach has been used for another neuronally enriched VGSC, $Na_v1.8$. The encoding gene, *SCN10A*, was screened in 104 individuals with diagnoses of idiopathic small fibre neuropathy with variants found in 10 of these (9.6%). When expressed in HEK293 cells, two of these mutations resulted in gain of function characteristics (Faber, Lauria, et al., 2012)**.**

A more recent publication screened 1139 patients diagnosed with pure small fibre neuropathy for variants in *SCN9A, SCN10A* and *SCN11A*. In this cohort, 'potentially pathogenic' variants were identified in 11.6%, harbouring 73 variants between them (5.1% in *SCN9A*, 3.7% in *SCN10A* and 2.9% in *SCN11A* (Eijkenboom et al., 2019). The significant majority of these variants were technically classed as 'variants of uncertain significance', according to Waxman's proposed classification of variants (Waxman et al., 2014) and, importantly, potential alternative causes of small fibre neuropathy were identified in a

majority of cases (including autoimmune diseases, glucose intolerance, diabetes, B12 deficiency, chemotherapy and alcohol excess). Some commentators even suggest that sodium channel gene analysis should form part of the work up for these otherwise idiopathic cases (de Greef et al., 2018).

Diabetic neuropathy is common but neuropathic pain is not always present. By sequencing *SCN9A* in individuals with confirmed diabetic neuropathy, with and without pain, Blesneac *et al.* identified 12 variants in 10/111 cases of painful diabetic neuropathy (and none in non-painful cases) (Blesneac et al., 2018)**.** As with the small fibre neuropathy studies by Faber *et al.* a number of these were either already known to exert gain of function characteristics or were shown to do so for the first time in this study, when electrophysiological experiments were performed in cells expressing these variants.

The same approach has been used for other causes of neuropathy including oxaliplatin (Sereno et al., 2017) and taxane induced neuropathy (Tanabe et al., 2020).

1.7.2 The concept of genetic risk in neuropathic pain

These studies raise an important question. Painful diabetic neuropathy is not an inherited condition. Whilst there may be an increased risk of developing diabetes if there is a family history (Florez et al., 2003), the condition is not a monogenic disorder like the very rare conditions discussed earlier. Similarly neuropathic pain in diabetic neuropathy is not an inherited condition although there is at least a degree of heritability (Meng et al., 2015). Genetic variance may be contributing to *risk* but certainly does not guarantee development

of either of these conditions. The penetrance of such variants would be said to be relatively lower than the mutations giving rise to PEPD, for example. If the individuals in Blesneac's study, in whom these variants were detected did not have diabetes, would they experience neuropathic pain anyway (which would then be termed idiopathic), or is it the interaction of the variant with the diabetes that results in neuropathic pain? In other words, could the variant be a risk factor for the development of neuropathic pain in a predisposing environment or situation? If one predisposing variant is identified, could there be others in the same individual such that combinations of different variants in different genes might confer greater or lesser risk of developing a phenotype? The finding of considerable phenotypic variability within a family harbouring exactly the same *SCN9A* mutation causing erythromelalgia reinforces the concept of relative risk (McDonnell et al., 2016) These questions have attracted considerable interest in the neuropathic pain field and are pertinent to the study of NFCI, given the clear environmental trigger and the predisposition of African individuals.

1.7.3 Genome wide association studies

With the advent of next generation gene sequencing, it is now easy to search for and identify particular genetic variants in any phenotype. Genome-wide association studies (GWAS) are a potentially powerful means to assess for genetic contribution/risk to/for phenotypic traits, particularly where a given trait is common and the genetic effect size is small. In this approach a large cohort of individuals with a given trait is compared, genetically, to a cohort without the trait and a profile can be generated of single nucleotide polymorphisms (SNPS) that are disproportionately found in the 'trait' group. To achieve sufficient power to identify variants with a small effect size, a very large number of genomes need to be sequenced, in

the order of tens or hundreds of thousands. This is especially problematic in a phenotype such as neuropathic pain because to achieve such numbers, data from many different studies may been to be combined. Different studies frequently use different definitions of neuropathic pain, and different methods of assessment, as found in a recent systematic review and meta-analysis by Veluchamy *et al.* (Veluchamy et al., 2018). The need for clear and consistent definition has been recognised in order for results from different studies to be meaningfully compared or incorporated. If this rigour is not undertaken variants may be missed, over-reported or conflicting results could be obtained. In the case of neuropathic pain, evidence based criteria for definition have been developed and widely adopted (Van Hecke et al., 2015).

It is equally important to define pathogenicity of variants in a systematic way. The American College of Medical Genetics has published guidance on how to ascribe different levels of pathogenicity to identified variants, such that these can be described as "pathogenic", "likely pathogenic," "uncertain significance," "likely benign," and "benign" (Richards et al., 2015). These guidelines are based on certain key factors including the frequency of the variant in the general population, previously published functional studies and the predicted effect of a variant on gene/gene product function.

These guidelines provide a framework, which can be applied in a uniform manner by different clinicians and researchers across the globe so that there is consistency in the description of pathogenicity.

Veluchamy *et al* performed a meticulous systematic review and meta-analysis of genetic risk factors for human neuropathic pain (Veluchamy et al., 2018). Through adherence to the PRISMA statement (Moher et al., 2009), the authors selected 29 high quality, pertinent studies (from 8052 identified in the preliminary literature search) to be scrutinised. Significant associations were found for 28 genes, including *SCN9A* and *SCN11A*. These were mainly involved in neurotransmission, immune response, and metabolism. Genetic variants in HLA genes, COMT, OPRM1, TNFα, IL6, and GCH1, were found to have an association with neuropathic pain in more than one study.

1.7.4 Genetic variance influences pain severity and analgesic responsiveness

The above discussion focusses only on genes associated with the presence (or absence in the case of CIP) of neuropathic pain. Many studies that were not included in Veluchamy's systematic review address the question of pain severity in disease as well as pain sensitivity in experimental pain paradigms (Duan et al., 2015; Reimanna et al., 2010). It is not surprising that variants in several of the genes already discussed are implicated. Examples include polymorphisms in *SCN9A* and pain severity in diabetic neuropathy (Li et al., 2015) sciatica and phantom limb pain (Duan et al., 2015; Reimanna et al., 2010). The inadequacy of our current pharmacological armamentarium (Finnerup et al., 2015) brings into focus not only the need for better agents, but also how drugs might be targeted to those most likely to respond (Fischer et al., 2009; Geha et al., 2016).

This discussion has focussed only on those genes most pertinent to human disease, particularly voltage gated sodium channel genes. Laboratory work and experiments in animal models have identified myriad additional candidates. The advantages and drawbacks

of such approaches have been reviewed (Calvo et al., 2019). At present the relevance of many these findings to human neuropathic pain is undetermined.

1.8 Transduction of innocuous, noxious and pathologic cold sensation

Cold allodynia is a striking feature of the late stages of non-freezing cold injury. It is helpful to consider the transmission of innocuous and noxious cold stimuli under normal circumstances to postulate how cold allodynia might arise. The following discussion is not intended as a review of the literature but will make several basic, but important points, of relevance to this thesis: 1) Cold sensing neurons are small diameter neurons, whose peripheral terminals are in the skin 2) At least some of these neurons are polymodal i.e. they respond not only to cold but also to mechanical stimuli 3) In the injured state, certain neurons may acquire a lower threshold for activation and 4) In the injured state, neurons previously activated only by noxious stimuli, may be activated by non-noxious cooling stimuli.

The pathways by which innocuous cool and noxious cold stimuli are detected and processed have been well reviewed (McKemy, 2013, 2018; Viana, 2018). Cold stimuli are sensed in the periphery by thinly myelinated Aδ fibres and by unmyelinated C fibres. This has been demonstrated in humans (Campero et al., 1996; Campero & Bostock, 2010; Torebjörk, 1974) and animals (Dubner et al., 1975; Simone & Kajander, 1997; Zotterman, 1936) using

selective nerve blocks and microneurographic recordings. The classification of these fibre types has been discussed earlier (Table 1.1).

The proportions of each fibre type and subpopulations in humans have not been established. Even in animals, the peripheral cellular encoding of innocuous and noxious cold perception is not fully understood. In particular there is conflicting evidence regarding the degree of specificity of cold sensing neurons for each of these stimuli, with some suggesting there is substantial overlap (Chisholm et al., 2018) and others that there is a much greater degree of specificity (Emery et al., 2016).

In addition to neural structures, it has recently been shown that non-neuronal keratinocytes (skin cells) are responsive to cooling. This is the case for rats, mice, ground squirrels and human cells. Furthermore, when keratinocytes are ablated in a mouse model, these animals display an increase in latency to withdrawal from a cold stimulus (Sadler et al., 2020). The biological importance of this putative thermosensing mechanism in humans is uncertain.

Following detection in the periphery, cold signalling is conveyed centrally. Afferent fibres synapse in the superficial dorsal horn, before, ascending in the spinothalamic tracts, to the thalamus and then to the insular cortex (Viana, 2018) As with all other sensory modalities peripheral input is integrated and modulated in the spinal cord by interneurons and descending pathways, respectively (Leith et al., 2010) . Cold and noxious cold are differentially processed in the human brain, as demonstrated by techniques including microstimulation (Davis et al., 1999), PET (Craig et al., 2000) functional MRI (fMRI) (Hua et al., 2005; Peltz et al., 2011) and magnetic encephalography (MEG) (Maihöfner et al.,

2002). These techniques have demonstrated many key, discrete brain regions involved in innocuous and noxious cold perception, including thalamus, insular cortex and somatosensory cortex.

1.8.1 Molecular detection and processing of innocuous and noxious cold

Our understanding of the molecular basis for detection of innocuous cooling, noxious cold and cold allodynia, is significant but incomplete. Recent reviews include: (Jordt et al., 2003; MacDonald et al., 2020; McKemy, 2013; Yin et al., 2015).

1.8.1.1 *TRPM8*

It was the pre-existing knowledge that a natural substance, menthol, was the 'activator' for an unknown cold sensor (Hensel & Zotterman, 1951), that in 2002 led to the identification of a molecular substrate for cold sensation, *TRPM8* (McKemy et al., 2002). Expressed on C fibre thermoceptors with terminals in the epidermis, the evidence for its role in innocuous cold perception is unequivocal (MacDonald, 2020) but this is unlikely be the only relevant molecular sensor of cold.

In mouse models where *TRPM8* is knocked out such animals consistently show an inability to detect innocuous cooling, and in several of these models they also show attenuated responses to noxious cold (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007; Knowlton et al., 2010). It is also clear, however, that *TRPM8* positive sensory afferents are not the only transducers of innocuous cold because when their cell bodies are labelled in dorsal root ganglia, they comprise only a proportion of neurons that are activated by cooling.

Only 32% of all cold sensing neurons in mouse trigeminal ganglia were activated by menthol in a recent study by Leijon *et al.* (Leijon et al., 2019)

In knockout animals abnormal behaviours include increased latencies to paw withdrawal from a cold plate, and a willingness to explore painfully cold surfaces in thermal preference tests (Colburn et al., 2007; Knowlton et al., 2010). Nevertheless, whilst responses to noxious cold are diminished, they are not absolutely abolished, and results between studies are highly variable, even using the same assay (McKemy, 2013). Bautista *et al* found that mice lacking *TRPM8* will avoid temperatures below 10°C, indicating that responses to noxious cold remain at least partially intact (Bautista et al., 2007). If *TRPM8* neurons are ablated *in vivo* with diphtheria toxin, whilst such animals do show impaired cold sensibility in the innocuous and noxious ranges they are still appear able to perceive noxious cold (Knowlton et al., 2013), which implies that additional molecular mechanisms are likely to be involved.

1.8.1.2 **TRPA1**

In 2003, an additional cold sensing TRP channel, *TRPA1*, was cloned (Story et al., 2003). The channel was found to be activated by noxiously cold temperature, and also by noxious chemical stimuli that cause a burning sensation (Bandell et al., 2004)**.** One such stimulus, cinnamaldehyde, elicited an identical current/voltage relationship to cold in Chinese Hamster Ovary Cells expressing the channel. When *TRPA1* is knocked out, or when neurons expressing *TRPA1* are ablated *in vivo,* however, results on behaviour are mixed. Some studies find that *TRPA1* deficiency results in no change in cold perception or noxious cold perception (Bautista et al., 2006; Knowlton et al., 2010), whilst profound deficits were

identified in another (Karashima et al., 2009). Data from *in vitro* experiments are similarly conflicting (Kwan & Corey, 2009). These starkly contrasting findings are not easily explained and cannot be due to species differences alone.

1.8.1.3 **Voltage gated sodium channels**

As already discussed, VGSCs have a vital role in human nociception, exemplified by the extreme phenotypes that may result from mutations, as well as evidence for a pathogenic role in commoner painful conditions such as small fibre neuropathy and painful diabetic neuropathy. Zimmerman *et al.* have shown that $Na_v1.8$, encoded by *SCN10A*, is required in mice for the ability to experience pain at low temperatures (Zimmermann et al., 2007), demonstrating that whilst tetrodotoxin sensitive VGSCs show enhanced inactivation at low temperatures, $Na_v1.8$ does not, and, furthermore, there is actually a reduction in activation for sodium currents through this channel at low temperatures. In concordance with this mice in which $Na_v1.8$ expressing neurons are ablated with diphtheria toxin show complete loss of noxious cold (Akopian et al., 1999) and mechanical sensation, whilst still being able to detect noxious heat (Abrahamsen et al., 2008). Cold pain thresholds are also significantly increased in $Na_v1.9$-null rats (Lolignier et al., 2015). Interestingly, behavioural responses to *TRPM8* and *TRPA1* agonists are not significantly different between $Na_v1.9^{-/-}$ and wildtype animals, implying that the mechanism through which cold pain is mediated by $Na_v1.9$ is independent of *TRPM8* or *TRPA1*. Furthermore, there is very little co-expression of either *TRPM8* or *TRPA1* with $Na_v1.9$ in mouse DRG (1.13% and 0.47%, respectively) (Lolignier et al., 2015).

Given the number of different channels for which compelling evidence exists, it seems likely that there is redundancy in the molecular processing of innocuous and noxious cold sensation. Indeed, this has been shown to be the case for noxious heat sensation (Vandewauw et al., 2018), and it is entirely plausible that a similar situation is true for cold.

1.8.2 Pathological cold pain has peripheral and central determinants

Cold allodynia is recognised in a variety of neuropathic pain states from both central (e.g. post stroke pain or multiple sclerosis) and peripheral lesions. Of the peripheral pathologies perhaps the best known example is as an acute manifestation of acute oxaliplatin-induced neurotoxicity (Staff et al., 2017), its presence predicting the development of a chronic, severe neuropathy (Attal et al., 2009). In contrast to the long-lasting cold sensitivity seen in NFCI, however, this is observed only during, and in the early period following (in the order of days), infusion. The phenomenon is well recognised in several other neuropathic conditions (Ochoa & Yarnitsky, 1994) including diabetic neuropathy (Callaghan et al., 2012), complex regional pain syndrome (Drummond, 2010), post traumatic injury (Collins et al., 1996) post shingles pain (Baron et al., 2009), and ciguatera toxin induced pain (Isbister & Kiernan, 2005) (Figure 1.6).

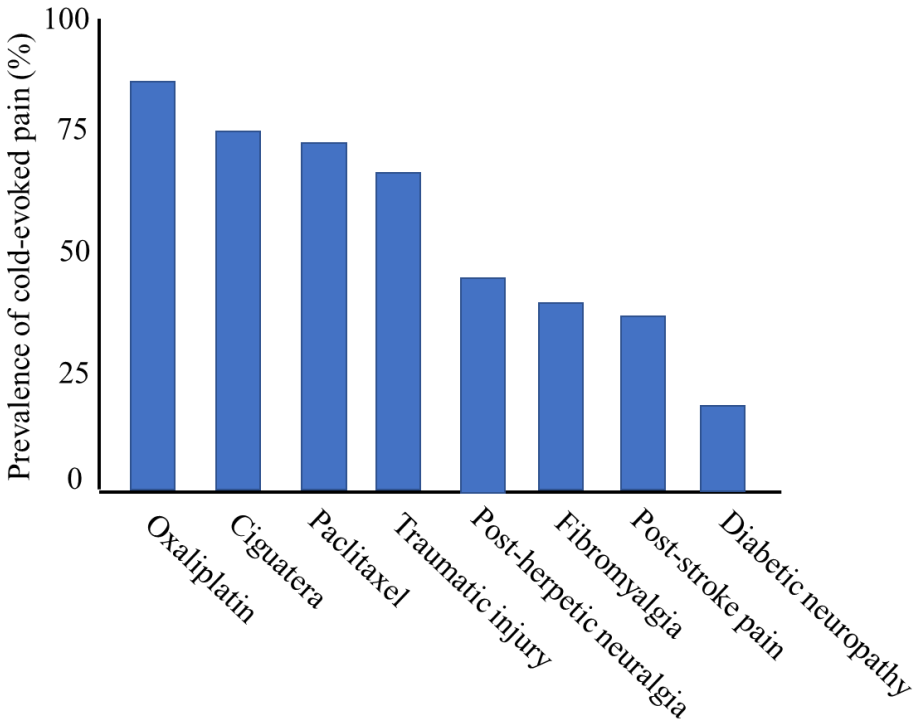


Figure 1.6 Average reported prevalence of cold-induced pain in human pain states (adapted from MacDonald et al., 2020)

The mechanisms by which neural injury induces a transition from innocuous cold to cold allodynia (or cold hyperalgesia) are not fully understood. Cold hyperalgesia and cold allodynia may arise from peripheral mechanisms, central mechanisms or both (Jensen & Finnerup, 2014; MacDonald et al., 2020; Yin et al., 2015)**.** Mechanisms may be at least partly aetiology specific as different experimental paradigms suggest different mechanisms. Similarly different mechanisms may be responsible in different species (Yin et al., 2015).

In the periphery cold allodynia could arise through 1) a lowering in the threshold of activation of cold nociceptors, such that innocuous cooling provokes firing, 2) the acquisition of a cold sensing phenotype by high threshold nociceptors, and evidence exists

for both of these mechanisms. Central mechanisms include a loss of tonic inhibition usually mediated by Aδ fibres or a loss of more central innocuous cold pathways in the brain (Jensen & Finnerup, 2014).

Given that understanding of non-pathological cold sensation is incomplete it is unsurprising that this is also the case for pathological cold sensation and that there are contradictions in the published literature. Whilst most of the evidence for these different mechanisms is derived from animal experiments studies in human point to both peripheral and central mechanisms.

Microneurographic recordings made from a patient with idiopathic small fibre neuropathy with cold allodynia provide direct evidence for a peripheral mechanism in humans (Serra et al., 2009)**.** Recording demonstrated abnormal spontaneous C fibre activity and responses to menthol, suggesting involvement of *TRPM8*. This is a report of just one patient and, moreover, she had previously undergone cervical and lumbar sympathectomies, which may confound interpretation of these results. Menthol application to human skin induces transient cold allodynia via sensitisation of C fibres (Wasner et al., 2004)**.**

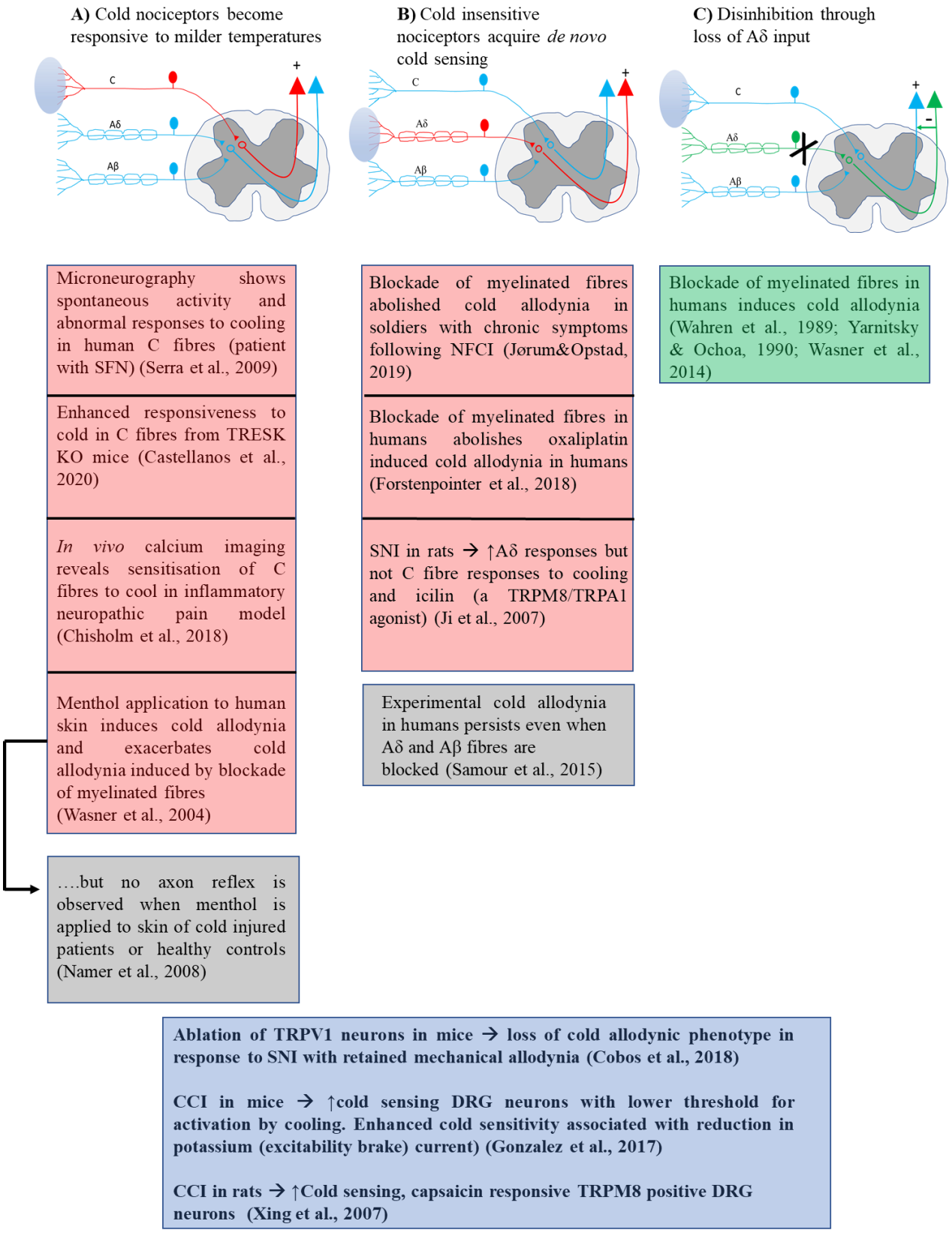


Figure 1.7 Postulated mechanisms for cold allodynia, with cited evidence. Adapted from Jensen & Finnerup (Jensen & Finnerup, 2014). Evidence in the blue box may favour either A or B. Evidence in grey boxes contradicts A and B, respectively.

Conversely, in favour of a central mechanism, a relatively simple observation that cold allodynia can be *induced* through selective blockade of myelinated fibres by compression, led to the theory that cold allodynia is, at least in part, due to a loss of tonic inhibitory input from myelinated fibres (Wahren et al., 1989; Yarnitsky & Ochoa, 1990). Assuming that such compression does not somehow cause hyper-excitability of C fibres, this would tend to suggest that there has been a change in central decoding of afferent input. Given that the ability to perceive cold was lost in these individuals, and yet burning pain could be elicited with innocuous cold stimuli, it was posited that cold sensing Aδ fibres might be a source of this tonic inhibitory input. This might explain why, in certain neuropathic pain patients, there is an impairment in the ability to perceive cold, and yet a seemingly paradoxical cold allodynia phenotype (Ochoa & Yarnitsky, 1994).

1.8.3 Mechanisms of cold allodynia induced by cold injury.

In contradiction to the findings of Wahren and Yarnitsky, an *abolition* of cold allodynia with blockade of myelinated fibres has also been observed in patients with cold allodynia following NFCI (Jørum & Opstad, 2019). This has also been observed in patients with cold allodynia following oxaliplatin (Forstenpointner et al., 2018).

Namer *et al.* applied menthol to cold allodynic areas of the skin of cold injured patients (Namer et al., 2008). Intriguingly, this did not significantly increase cold allodynia relative to healthy controls. Some individuals actually experienced an improvement in allodynia. No axon reflex flare was observed in response to menthol in either cold injured participants or controls, arguing against an upregulation or *de novo* expression of TRPM8 on mechanoinsensitive C fibres as a mechanism of cold allodynia

In an experimental paradigm where mild freeze injury was applied to the skin of rats, electrophysiological recordings were made from neurons in the superficial dorsal horn, with neurons categorised as wide dynamic range or high threshold based on responses to thermal and mechanical stimuli within their receptive fields. After injury there was a dramatic lowering of threshold to subsequent warm and cold stimuli (Khasabov et al., 2001). For example, before freezing injury the mean cold threshold for firing of high threshold neurons was 0.3°C, which decreased significantly to 10.6°C afterwards. The size of the response was also augmented. The authors also noted a *de novo* cold sensing in neurons that had previously only reacted to heat. As recordings were only made from dorsal horn neurons, and not from peripheral nociceptors, it is difficult to discern whether this enhanced responsiveness was due to sensitization of nociceptors or central neurons.

Cold allodynia has been studied at the level of the brain, in humans, by fMRI. Following the temporary induction of cold allodynia through application of menthol to the skin, the investigators found that in the allodynic state, in addition to those brain areas usually activated by noxious cold, there was additional activity in the dorsolateral prefrontal cortex, and midbrain (Seifert & Maihöfner, 2007). This seems unlikely to be a specific effect as the DLPFC is well known to be abnormally active in a variety of experimental pain paradigms, as well as basally in chronic pain patients (Seminowicz & Moayedi, 2017)

1.8.4 Molecular basis of cold allodynia

There is much evidence that changes in the expression of several different molecular species within neuronal subtypes, underlies the development of cold allodynia. It is unsurprising that there is substantial overlap with those molecules whose roles in non-pathological cold sensation have been demonstrated. Particularly important molecular families include TRP channels, sodium channels and potassium channels, which have been recently reviewed by Macdonald (MacDonald et al., 2020).

It is likely that the mechanisms responsible for cold pain/allodynia in one condition may be different from those in another. Results are conflicting, possibly due to different models of nerve injury (e.g. different toxins such as oxaliplatin or ciguatera, and different methods of mechanical injury such as ligation versus constriction) and the use of different behavioural assays to assess for cold allodynia, with different stimuli used, and different durations of cold exposure (Kwan & Corey, 2009; MacDonald et al., 2020; Yin et al., 2015).

1.8.4.1 **TRPM8**

The evidence for role of *TRPM8* in cold allodynia is mixed, much of it derived from the same sets of experiments demonstrating its importance in noxious cold sensation. In animal models the presence of cold allodynia can be assessed by observation for behaviours such as paw licking and shaking in response to innocuous cold stimuli. Animals lacking *TRPM8*, or in which *TRPM8* expressing neurons are ablated show attenuated cold hyperalgesia in a variety of nerve injury models, including sciatic chronic constriction injury (Colburn et al., 2007) and an inflammatory model where CFA is injected into a paw (Knowlton et al., 2013).

However, although attenuated, they do still exhibit aversive responses, both compared to the pre-injury state, and when the injured side is compared to the non-injured side, suggesting an additional mechanism.

Antagonists to *TRPM8* alleviate the cold allodynia seen in a variety of nerve injury models, including the sciatic chronic constriction injury (Calvo et al., 2012; Cao et al., 2019; Xing et al., 2007) and spinal nerve ligation model (Patel et al., 2014)**.**

1.8.4.2 **TRPA1**

Despite the evidence implicating *TRPM8* in pathological cold sensation, there are no reported human conditions in which *TRPM8* is mutated. In contrast *TRPA1*, is mutated (N855S) in a human monogenic paroxysmal extreme pain disorder where pain is triggered by cold. Cultured neurons expressing the N855S mutation show gain of function characteristics on voltage clamp experiments compared to WT. (Kremeyer et al., 2010). Further evidence for a role of *TRPA1*, and of potential clinical relevance, is derived from an oxaliplatin mouse model. In mice lacking *TRPA1* the cold allodynic effect of oxaliplatin is lost. The authors of this study also demonstrated that in wild type rats, oxaliplatin induced cold allodynia was blocked by the administration of a *TRPA1* antagonist (Nassini et al., 2011)**.**

1.8.4.3 **Voltage gated sodium channels**

Several observations suggest that there is likely to be a role for VGSCs in cold allodynia. As previously mentioned, very rare mutations in *SCN11A*, encoding $Na_v1.9$, result in a CIP-like phenotype, where temperature sensation in the innocuous range is maintained, but where an unpleasant sensation is evoked by variation in temperature, such as a gust of wind (Woods et al., 2015)**.** The VGSC, $Na_v1.6$ appears to be important for the generation of cold allodynia in a mouse model of oxaliplatin induced neurotoxicity, whereby if $Na_v1.6$ channel function was abrogated using selective inhibitory molecules, cold allodynia was completely abolished (Deuis et al., 2013). In animals lacking either $Na_v1.8$ or $Na_v1.9$, no effect on the development of cold allodynia following oxaliplatin was observed (this is surprising given Zimmerman's findings that $Na_v1.8$ is absolutely required for mice to experience pain at low temperatures (Zimmermann et al., 2007). There is also conflicting evidence from another group, showing that disruption of $Na_v1.9$ expression in rats *does* alleviate cold allodynia associated with oxaliplatin (Lolignier et al., 2015)). In contrast, in a model of ciguatera induced cold allodynia, the authors observed that administration of a selective $Na_v1.8$ antagonist inhibited ciguatoxin-induced cold hypersensitivity (as derived from measurement of excitability of dorsal horn neurons rather than any behavioural assay) (Patel et al., 2015).

1.8.4.4 **Potassium channels**

One further mechanism implicated in cold allodynia is via the potassium current excitability brake, which is mediated by both voltage gated Shaker related potassium channels of the *KCNA* family (González et al., 2017; Memon et al., 2017) and background, or "leak" potassium channels (potassium two pore channels or K2P) of the *KCNK* family (MacDonald et al., 2020). A variety of techniques including skin-nerve preparations, calcium imaging and behavioural assessment of knockout animals, demonstrate that removal of the currents

conveyed by these channels results in hyperexcitable nociceptive phenotype, with neurons acquiring new cold sensitivity as well as higher temperatures for activation, consistent with cold allodynia (Castellanos et al., 2020).

Oxaliplatin has been shown to exert its cold allodynic effect not only via TRP channels and sodium channels, but members of the two-pore domain potassium channel family also appear to have an important role. In mice Oxaliplatin significantly lowers the expression of distinct potassium channels, including TREK1, TREK2, and TRAAK channels (Descoeur et al., 2011; Pereira et al., 2014) and null mice show abolished oxaliplatin-induced cold hypersensitivity. Furthermore, Descoeur *et al.* found that not only was there a reduction in the expression of TREK1 and TRAAK, but also that there was a significant increase in the expression of HCN (hyperpolarisation activated cation nucleotide) channels, which are inwardly rectifying, acting to limit the extent of hyperpolarisation (Descoeur et al., 2011).

1.8.4.5 Pathological cold sensation is unlikely to derive from one molecular species alone

The available evidence does not localise pathological cold sensation to any one specific transduction molecule, but rather suggests that it arises from an unfavourable redistribution of various ionic channels in a small subset of sensory neurons (Viana et al., 2002). Furthermore, the available evidence suggests that cold allodynia arises through different molecular mechanisms depending on the nature of neural insult (Yin et al., 2015) and the contribution of the central nervous system adds another world of complexity.

1.8.5 The aims of this thesis

1) To define the neurological phenotype in greater detail than has been done previously. A contemporary study is required, using modern techniques, as the phenotype seen now is more subtle than that observed for much of the 20th Century.
2) To use contemporary, evidence-based guidelines, to establish if the pain experienced in 21st Century cases is neuropathic in nature.
3) To ascertain whether there may be any genetic risk. As already discussed, neuropathic pain has a significant heritable component. There is considerable interest in the identification of genetic variants as risk factors, interacting with environment, to bring about neuropathic pain states.
4) To use threshold tracking, a technique for the assessment of axonal excitability, to investigate potential pathophysiological mechanisms.

2 The Neurological Phenotype of NFCI

2.1 Introduction

2.1.1 Neurological symptoms and signs are described in NFCI

The most detailed clinical descriptions of cases of NFCI are derived from observations made during, and shortly after, major conflicts, in particular the First and Second World Wars. At those times the condition was variously known as “Trench Foot”, “Immersion Foot”, “Trench Frostbite” and “Vasoneuropathy” after chilling (Smith et al., 1915; Ungley et al., 1942; Ungley et al., 1945). In addition to the visible skin changes observed there are clear descriptions of (predominantly) sensory symptoms suggesting neurological involvement, both in the early phases of the condition and much later on. Whilst the descriptions are detailed, and with vivid language used, these publications suffer from both the lack of a systematic approach and the absence of the quantitative techniques now at our disposal for the investigation of sensory dysfunction.

Ungley was the first author to define different phases of NFCI, which he termed 1) prehyerpaemic, lasting hours to days, 2) hyperaemic, lasting 6-10 weeks, 3) post hyperaemic lasting weeks or months and 4) “late sequels”, symptoms ongoing into the second year post injury (Ungley, 1942). This thesis is concerned with the investigation of the chronic pain experienced by a proportion of those affected by NFCI. In the field of neuropathic pain, the definition of ‘chronic’, is pain that is persistent or recurrent for ≥ 3 months (Scholz et al., 2019), corresponding to Ungley’s posthyperaemic and “late sequels” phases.

2.1.2 The Chronic Phenotype (posthyperaemic and late sequels)

Few detailed studies have been published specifically addressing the sequelae of cold injury (either freezing *or* non-freezing) after this duration. It remains unknown what proportion of those injured by NFCI will progress to have chronic symptoms. In Ungley's "post hyperaemic" phase, which lasts weeks to months, pains and tingling (usually) improve and sensation returns to normal. He notes, however, that recurrences are not uncommon, even years after exposure. In relation to 18 patients surveyed 2 years following injury, sequels mostly included transient tingling, aching and stabbing pains, usually related to prolonged standing or exposure to cold and wet conditions. However, it is not stated what proportion of cases enter this phase, and detailed neurological examinations are not systematically documented (Ungley, 1942).

Prior to the commencement of this thesis, the most detailed descriptions of late sequelae were published in 1946 by Paddock *et al* (Paddock, 1946) and Abramson *et al* (Abramson et al., 1946). The Paddock publication presents findings from cases of "mild to moderate" (undefined in the manuscript) trench foot sustained in the Apennine Mountains in the winter of 1944. It is not specified how many cases were assessed overall. The chief complaints are documented as spontaneous pain in the feet and tenderness of the soles. Vibration sense and joint position sense were tested in 33 individuals, with deficits seen in 8 (24%) and 13 (39%) cases, respectively. Furthermore, using test tubes containing cool and warm water, experiments were conducted testing temperature discrimination. This methodology, reminiscent of a rudimentary form of QST, revealed an inability to distinguish temperature in all cases tested, in contrast to a group of healthy controls, all but one of whom were readily able to distinguish the temperatures. Interestingly immersion of affected feet into ice water

evoked aching in only one of ten patients, compared to ten of ten healthy control participants. The interpretation of this finding was that there must have been injury to C fibres, as these were known to mediate noxious cold, and it was concluded that in these patients "the terminal nerve twigs are at fault – a terminal neuritis"

In the much larger study undertaken by Abramson et al, reporting on 633 cases of trench foot, the vast majority (548, 87%) did not suffer any loss of tissue. This is pertinent as, in contrast to Trench Foot and Immersion Foot, gangrene and loss of tissue is rarely reported in contemporary cases of NFCI (Kuht et al., 2018). In addition, these cases were more chronic than those reported by Paddock, assessment taking place 13 months post injury. Abramson classified his patients into three broad groups: those with predominant signs of excessive sympathetic activity, those with signs attributable to peripheral nerves ("but with few objective findings"), and a third group with subjective and objective clinical findings. Symptoms included "aching (3.8%), tenderness in the sole of the foot (13.6%), numbness 16.6%), neuritic pains (14.3%), hypaesthesia (41.5%) and burning and tingling (21.9%)". Regarding the second group of patients, those with "signs attributable to peripheral nerves", a slightly contradictory statement is made that "objectively there were very few abnormalities noted" but later in the same paragraph "many of these individuals were found to have areas of hypesthesia to pinprick and cotton wool". The exact numbers of patients with sensory disturbances are not reported and instead a table is devoted to other signs such as abnormality of gait, atrophy of muscles, stiffness of toes, coldness and oedema. Questionnaires were completed by 29% of the soldiers 3 months after returning to civilian life (only 33% of the whole cohort were able to retain some sort of employment in the army following hospital discharge). The table below (Figure 2.1) summarises the results (Abramson et al., 1946)

TABLE IV. EVALUATION OF PROGRESS OF PATIENTS APPROXIMATELY THREE MONTHS AFTER RETURN TO CIVILIAN LIFE

A. Types of Occupation			
TYPE	% OF CASES	TYPE	% OF CASES
Farming and gardening	15.4	Factory worker	30.5
Cab or truck driving	14.6	White-collar job	24.0
Machinist or helper	15.5		
B. Symptoms Which Persist in the Feet			
SYMPTOMS	% OF CASES	SYMPTOMS	% OF CASES
Burning sensation	72	Tenderness, sole of foot	69
Hyperhidrosis	63	Dermatophytosis	28
Blister formation	26	Swelling	59
C. Ability to Walk			
DISTANCE	% OF CASES	DISTANCE	% OF CASES
Less than ¼ mile	12.9	¾ mile	8.7
¼ mile	12.1	1 to 1½ miles	33.7
½ mile	17.2	2 to 3 miles	15.4

Figure 2.1 Taken from Abramson et al. (Abramson et al., 1946), a table recording employment and symptoms at 3 months following return to civilian life

Redisch et al (Redisch et al., 1951) reported 100 cases of "chronic trench foot", with similar findings to Abramson. Namely complaints of pain, numbness, tingling, burning and anaesthesia were common. Redisch observed that "all patients felt decidedly better during the warm weather and reacted promptly to a cold spell with increase in subjective complaints (including pain and numbness) and decrease in work tolerance". All these 100 cases reported a severe worsening of pain and "impairment of work tolerance" on exposure to cold conditions. The patients' symptoms are all attributed to vascular pathology, partly on the basis that no neurological deficit is present. However neurological examination is not described anywhere in the publication, unlike Paddock's series 5 years previously.

Blair et al (Blair et al., 1957) reported on the symptoms 100 patients, four years following freezing cold injury (i.e. frostbite) sustained in Korea. Pain and numbness of the feet were

present more frequently during winter months. Despite no neurological examination having been examined the authors stated that "all symptoms are more severe than organic physical findings would indicate" and that "monetary compensation from the Veterans Administration may be involved." However, as observed by Paddock *et al* "only on neurologic examination of the distal portions of the feet does any abnormality become apparent" (Paddock, 1946), and it appears that little importance was attached to these findings that we see so often today in patients with small fibre neuropathies of many different aetiologies.

A series of 40 Norwegian soldiers was described by Rosen *et al.*, who had sustained cold injuries during service, with an exacerbation of symptoms, described as mild to moderate in severity upon re-exposure to the cold (Rosén et al., 1991)**.** Helpfully the authors provide a definition of cold sensitivity as evocation or exacerbation of pain, numbness or paraesthesia within a shorter period of time upon cold exposure compared to before injury.

Arvesen *et al* (Arvesen et al., 1996) reported abnormalities of nerve conduction studies even several years after 'local cold injury'. Although it is not explicitly stated whether these were freezing or non-freezing cold injuries, participants' injuries were stratified according to criteria developed for frostbite, and therefore presumably these participants had suffered frostbite rather than NFCI.

Carlsson *et al* reported on a small cohort of 15 military recruits in the Swedish army, who had sustained *freezing* cold injuries during their training (Carlsson et al., 2014). Questionnaire and QST data were presented from 4 months post injury. Pain was reported

on a numerical rating scale from 0-10, with average scores, in the feet, of 2.6 at 4 months. The questionnaire was re-administered at 4 years post injury, with an average pain intensity of 2.5; relatively mild, but persistent, nonetheless. Even after 4 years, 6 out of 8 participants reported "quite a lot" of pain and discomfort when exposed to the cold. The QST measures included vibration perception, and thermal thresholds. Interestingly 5 out of 6 conscripts who had sustained injuries to their hands but *not* their feet, nonetheless showed significant abnormalities of vibration and thermal detection in the feet. This may indicate subclinical neuropathy.

A larger, prospective study, also performed by Carlsson et al (Carlsson et al., 2016), reported QST findings at baseline *and* after 14 months of cold weather army training. Statistically significant losses of function were revealed for thermal thresholds and for vibration detection. Only 8 of the cohort of 81 soldiers reported sustaining a cold injury (defined as an affirmative response to the question, "have you developed frostbite during your military training?". Therefore, a number of cases of NFCI could have been missed. This is reinforced by the finding that 22 participants reported pain on exposure to cold *after* their training, compared to 0 *prior* to their training). These were reported to have even greater abnormalities than those who had not sustained an injury, but these data were not presented in the publication.

Most recently, Jorum (Jørum & Opstad, 2019) described a case series of 26 Scandinavian naval soldiers with follow up data up to 4 years following injury in 7 of these participants. All of these complained of ongoing pain in the extremities upon exposure to cold.

Furthermore, these soldiers all displayed gain of function for cold pain on thermal threshold testing.

2.1.3 Deep Phenotyping in the Investigation of Neuropathic Pain

Deep phenotyping is a comprehensive, multimodal approach, enabling detailed characterisation of the clinical presentation and nature of disease processes. In the case of neuropathies this includes structured clinical assessment, a battery of questionnaires and tests aimed at interrogation of the sensory nervous system, including nerve conduction studies, quantitative sensory testing and skin biopsy. This methodology has been used to investigate the pattern of sensory nerve dysfunction in a variety of painful sensory neuropathies, including diabetes (Themistocleous et al., 2016)**,** HIV (Phillips et al., 2014) and heterozygote women with Fabry Disease (Torvin-Møller et al., 2009)

2.1.4 The Grading of Neuropathic Pain

Neuropathic pain is that which arises as a direct consequence of a lesion or disease affecting the somatosensory nervous system (Jensen et al., 2011). The most recent iteration of the Neuropathic Pain Special Interest Group (NeuPSIG) of The International Association for the Study of Pain (IASP), provides clear guidance on the requirements to make this diagnosis (Finnerup et al., 2016). For neuropathic pain to be considered ‘definite’, four criteria must be satisfied: 1) history of a relevant lesion, 2) a plausible neuroanatomical localisation of pain, 3) examination findings corresponding to this localisation and 4) objective demonstration of the lesion by a confirmatory test.

The pain reported by authors in the first half of the 20th Century was described as "neuritic" but without consistent or systematic reference to its localisation, examination findings or confirmatory tests. No criteria were referred to when describing pain as neuritic, but rather this term was used in a more colloquial sense. Nevertheless, pain was described as present in the distal extremities and examination findings in this same distribution were also described. It is not reported what proportion of patients with chronic pain had abnormal examination findings, or to what extent. In terms of confirmatory tests quantitative sensory testing, microneurography, laser/heat evoked potentials, skin biopsy for determination of IENFD and genetic testing had not been developed. Nerve conduction studies were reported to be abnormal in some of Ungley's cases (Ungley, 1942) but again, it is not reported how many patients, which tests were performed or the clinical phenotype of the patients who had abnormal recordings. The coincidence of pain in the feet, abnormal examination findings and "impaired electrical responses" in one patient is depicted in a progress chart (Figure 1.1). This one patient would satisfy contemporary criteria for definite neuropathic pain. However, such charts were not published for all patients.

Interestingly there is precedent for the retrospective application of 21st Century neuropathic pain grading criteria to a historical cohort (Roocroft et al., 2017). The authors interrogated the case records of Far East Prisoners of War (FEPOW) suffering from so-called Burning Feet Syndrome, probably resulting from malnutrition in captivity. Despite careful, contemporaneous documentation of neurological findings by a fellow captive, the authors were only able to make a diagnosis of "possible" neuropathic pain in most patients.

Despite the publication of several case series of patients with chronic sequelae of cold injury, it is difficult to ascertain the likelihood that any pain present was neuropathic. There are two particular reasons for this: 1) there is a lack of detail regarding clinical assessment in terms of pain localisation and characteristics and 2) diagnostic tests were lacking at the time the studies were undertaken, especially those targeting small nerve fibres. Table 2.1 summarises the available literature in respect of those factors required to grade pain following cold injury as neuropathic (relevant insult, plausible distribution of symptoms, concordant examination findings and diagnostic tests).

Publication	Setting of injury	Time since injury	Cohort size	Symptoms	Signs	Tests
Paddock 1946	Apennine Mountains Winter 1944 (Trench Foot)	2-3 months	Unspecified	Spontaneous pain. Difficulty walking due to pain	Hypalgesia to pinprick in great toes in "almost all cases". 13/33 poor JPS and 8/33 absent vibration at great toe. Impaired temperature discrimination at great toe in 14/14 cases	None
Abramson et al., 1946	Nov 1943-March 1944 Aug 1944-Feb 1945. (Trench Foot)	4-13 months	633	Of 596 "mild cases": numbness in 16.6%; "neuritic pain" in 14.3%; burning and tingling 21.9%; aching 3.8%. After discharge to civilian life 72% of 250 reported ongoing burning	Of 596: Abnormal gait 34%; Atrophy of foot muscles 14.7%; "Hypaesthesia" 41.5%	None
Redisch et al., 1951	European Campaign, Winter 1944-1945	5-6 years	100	Sensitivity to cold 100% Pain on walking 91% Pain at rest 32% Tingling 93% Burning 96% Anaesthesia 3%	"Absence of neurological findings". Examination not documented	None
Blair et al., 1957	Korea, Winter 1950-1951	4 years	100 approached. 97% completed questionnaires. 89% further assessed	Pain in summer months 43% Pain in winter months 67% Numbness in summer months 30% Numbness in winter months 68%	"All symptoms more severe than organic physical findings would indicate." No neurological examination documented	None
Oakley, 1996	Choisin Reservoir, Korea 1951. (Mixed freezing and NFCI)	45 years	101	Pain 59% "Cold sensitivity" 88%	Sensory loss in feet in 22%	None
Golden et al., 2013	Falklands Conflict, 1982 (NFCI)	3 months	1505/2354 who completed questionnaires had acute symptoms of NFCI. 154/1505 had ongoing symptoms at 3 months	Range from asymptomatic to "overt neurological symptoms"	Evidence of "mild peripheral nerve damage". No documentation of examination findings	"A range of impaired nerve conduction studies in 35%" - undefined
Carlsson et al., 2014	Northern Sweden, Winter 2007	4 months and 4 years	15	Pain and cold hypersensitivity	None documented	QST: Abnormalities of temperature and vibration detection

Table 2.1 A summary of the literature pertaining to late sequelae of cold injury in general, prior to the commencement of this work

2.2 Methods

2.2.1 Study participants

Study participants were enrolled into one of two studies. Actively serving British army personnel were enrolled in the "Neurological consequences of non-freezing cold injury" study approved by the Ministry of Defence Research Ethics Committee (MoDREC Protocol No: 616/MoDREC/14). Ex-serviceman (veterans) were enrolled in the "Pain in Neuropathy Study (PiNS)" (Themistocleous et al., 2016), approved by the National Research Ethics Service of the United Kingdom. (No.: 10/H07056/35). Study participants were recruited from a specialist neuropathy clinic at The John Radcliffe Hospital, Oxford UK, or through self-referral via advertising. Control participants, matched for age and cold exposure were recruited from Catterick Garrison, North Yorkshire. Advertisements were placed at locations on the garrison.

To be eligible participants had to be over the age of 18 and have a history consistent with chronic NFCI. This was defined as: sensory symptoms (numbness, paraesthesias, pain and altered temperature sensibility) that began at the time of exposure to a near freezing cold environment and persisted for at least three months, with no history of soft tissue freezing that would indicate a diagnosis of frost bite or frost nip. Exclusion criteria included history of a medical condition that may cause a neuropathy; pregnancy; poor or no English language skills; severe pain at recruitment from a cause other than NFCI (to prevent potential confounding influence on pain reporting as well as psychological and quality-of-life reported outcomes); patients with documented central nervous system lesions; coincident

major psychiatric disorders; or patients with insufficient mental capacity to obtain informed consent or to complete questionnaires.

To be enrolled as a control participant, individuals had to have no symptoms of neuropathy and no other sources of pain. They had to be otherwise healthy with no history of any neurological condition. They had to have undergone similar military training to NFCI participants and to have been exposed to cold conditions for prolonged periods without any lasting symptoms lasting longer than 24 hours following this/these exposure(s).

2.2.2 Assessment of study participants' symptoms and examination findings

The study design consisted of a clinical assessment and investigations, quantitative sensory testing (QST), nerve conduction studies and skin biopsy, which were completed in one or two visits at the John Radcliffe Hospital, Oxford, UK. The clinical assessment consisted of a detailed history and structured neurological examination. A narrative of the study participant's injury, including the environment where the injury was sustained, the nature of symptoms at onset and evolution of symptoms since injury, and impact the injury had on employability were recorded in a standardised manner. The participants also completed validated and standardised questionnaires that recorded the nature of their pain and the impact on their quality of life. All participants underwent screening for other potential causes of neuropathy.

2.2.2.1 **Questionnaires**

The Douleur Neuropathique en 4 Questionnaire (DN4) (Bouhassira et al., 2005) and the Brief Pain Inventory (Tan et al., 2004) were completed to characterise symptom profile and any limitations caused by these symptoms. The DN4 is a screening tool for neuropathic pain. The BPI is a two-part questionnaire tool validated for assessing the severity of, and interference caused by, pain. These were completed at the time of clinical assessment. Participants were also invited to complete the Short Form Health Survey (SF-36) (measuring health related quality of life across several domains), the Insomnia Severity Index (ISI), the Pain Anxiety Symptom Scale (PASS), the Depression, Anxiety and Positive Outlook Scale (DAPOS) and Pain Catastrophising Scale (PCS) questionnaires

2.2.2.2 **Clinical assessment**

Structured motor and sensory neurological examination was performed in all patients to detect signs of a peripheral neuropathy (Compston, 2010; Kleyweg et al., 1991). The examination included assessment of muscle bulk, motor power, deep-tendon reflexes, temperature sensibility, light touch and pinprick sensation, proprioception, and vibration perception (Phillips et al., 2014). These assessments were made independently from the performance of QST. Dorsalis pedis and posterior tibial pulses were assessed.

A sensory sum score was calculated for each patient, whereby a score was given for each of the four limbs and for each of four modalities: pinprick sensation, light touch sensation, vibration sensation and joint position sense/proprioception. Each of the first three modalities was first tested over the sternum and participants were asked to use this as a reference when sensation in the limbs was tested. Vibration was assessed as either present or absent rather than attempting to score the degree of deficiency, which is assessed separately in the QST

protocol. Temperature sensibility was not included in the sensory sum score. Scores were assigned as follows: 0 = no abnormality; 1 = abnormality at the toe/ finger; 2 = abnormality at the ankle/wrist; 3 = abnormality at the knee/elbow; 4 = abnormality at the hip/shoulder. The maximum possible score is 64 (a score of 4 for each of the four modalities in each of the four limbs). Therefore, the sensory sum score quantifies the severity of sensory abnormalities with a higher sum score indicative of greater sensory loss.

2.2.3 Determination of intraepidermal nerve fibre density

2.2.3.1 Procurement of biopsy material

Intra-epidermal nerve fibre density (IENFD) assessment from skin biopsy specimens is a validated and sensitive test for the detection of small fibre pathology. Punch biopsies (3mm) of the skin were taken in accordance with the consensus document produced by the European Federation of Neurological Societies/Peripheral Nerve Society Guideline on the utilisation of skin biopsy samples in the diagnosis of peripheral neuropathies (Lauria et al., 2010)

Skin biopsies were not taken from participants on warfarin or those who had other contraindications to skin biopsy. Biopsies were taken 10 cm proximal to the lateral malleolus with a disposable 3-mm punch biopsy circular blade (Stiefel Laboratories Inc.) after subcutaneous lidocaine (1%, 1–2 ml) administration.

2.2.3.2 Processing of biopsy material

The biopsy was fixed in fresh periodate-lysine-paraformaldehyde (2%) for 12 to 24 h. Tissue was then washed in 0.1 M phosphate buffer and stored for 2 to 3 days in 15% sucrose in 0.1 M phosphate buffer. After embedding in O.C.T. (Fisher Scientific), the tissue was snap-frozen and stored at -80C. Skin sections with 50-mm thickness were cut on a cryostat, and

immunohistochemistry for protein gene product 9.5 was performed on free-floating sections using the immunoperoxidase method. Staining was performed on 24-well plates allowing reagents complete penetration of floating samples. For the bright-field method, samples were washed with Tris-buffered saline (TBS) and placed on a 5% hydrogen peroxide solution on ethanol, followed by blockade of non-specific protein binding with 4% normal donkey serum, 0.5% milk, and 0.1% TritonTM X in TBS. Primary antibody rabbit anti-protein gene product 9.5 (1:15 000; Ultraclone Ltd, or 1:800, Zytomed, Systems) was incubated overnight at room temperature. After rinsing the samples with TBS, secondary biotinylated goat anti-rabbit IgG antibody (1:400; Vector Laboratories) was used followed by addition of VECTASTAIN ABC Kit (Vector Laboratories) for 1 h. Samples were washed and transferred to the DAB Peroxidase Substrate Kit, 3,3'-diaminobenzidine (Vector Laboratories) until a visible stain emerged. Samples were rinsed with distilled water and progressively dehydrated with ethanol. Samples were mounted using DPX mounting media.

2.2.3.3 **Measurement of IENFD**

Protein gene product 9.5-immunoreactive nerve fibres crossing the basal membrane of the epidermis were counted under a 40-x objective, and a measurement of the length of the sample was also obtained. The IENFD was assessed using a double bright-field microscope using established counting rules and expressed as fibres per millimetre of epidermal length, and considered abnormal if the value falls below the fifth centile for age- and gender-matched healthy controls (Lauria et al., 2010a). NFCI participants' biopsies were performed, processed and analysed at The John Radcliffe Hospital, Oxford. Cold exposed control participants' biopsies were performed at Catterick Garrison, North Yorkshire but processed and analysed at The John Radcliffe Hospital, Oxford. Participants' IENFDs were determined to be normal or abnormal relative to the published normative age and gender matched data

(Lauria et al., 2010a). Values falling below the 0.05 quantile are abnormal. There are no published normative skin biopsy data for individuals of African descent. Age, gender, and ethnically matched skin biopsies from healthy African Americans were obtained and analysed by The Cutaneous Nerve Laboratory of The John Hopkins School of Medicine, Baltimore, USA. Healthy study participants were recruited through a John Hopkins School of Medicine approved protocol (NA_00036876) and screened for causes of neuropathy. All healthy participants were examined and determined to have a normal peripheral nerve exam and there was no history of diabetes, thyroid disease, cancer, HIV, hepatitis, vitamin B12 deficiency or significant alcohol intake (42 units/day). Immunohistochemistry was performed using mouse anti-human monoclonal anti protein gene product 9.5 (1:2500; Bio-Rad). IENFD assessment was performed by Kelly Byrnes (K.B.) at The Johns Hopkins School of Medicine, Baltimore, and by Dr Andreas Themistocleous (A.C.T.) and Dr Tom Vale (T.V.) at The John Radcliffe Hospital, Oxford. Reliability analysis performed by Johns Hopkins, shows that Zytomed antibody, used by John Radcliffe Hospital, and Bio-Rad antibody, used by John Hopkins, are equivalent, with R2 = 0.92. Reliability analysis was performed to assess IENFD assessment between A.C.T., Oxford, and K.B., Baltimore, assessing counts in 11 different individuals with each investigator blind to the other's scores. Intra-class correlation coefficient, expressed as mean [95% confidence interval (CI)], was 0.97 (0.88–0.99) with R2 = 0.94. Therefore, the agreement between the laboratories was very good.

2.2.4 Nerve conduction tests

Nerve conduction tests were performed in line with the American Academy of Neurology and American Association of Electrodiagnostic Medicine recommendation for the diagnosis of a peripheral neuropathy (England et al., 2005). The nerve conduction tests were

performed with an ADVANCE system (Neurometrix) or a Medelec Synergy EMG system (version 15.0; Viasys Healthcare UK Ltd) using conventional reusable surface recording electrodes. Sural sensory and peroneal motor nerve conduction studies were performed in one lower extremity. If both studies were normal, no further tests were performed (Buschbacher and Orahlow, 2006). If either test was abnormal, additional nerve conductions were performed that included: ipsilateral tibial motor nerve, contralateral sural sensory nerve, peroneal motor or tibial motor nerves. The minimum case definition criterion for electrodiagnostic confirmation of neuropathy was an abnormality of any attribute of nerve conduction in two separate nerves, one of which was the sural nerve. Variables such as skin temperature, age, height, sex, and weight were measured and accounted for when interpreting nerve conduction tests. Sympathetic skin responses were measured with patients lying supine with eyes closed in a quiet room with skin temperature at 32°C or above. Disposable Ag/AgCl electrodes were attached to the mid-palm of one hand and the mid-sole of one foot and referenced to the corresponding dorsal surface. A single 0.2 ms duration 200–300 V square-wave electrical pulse was applied to the contralateral median/tibial nerve (separately for upper and lower limbs, respectively) after an unpredictable interval to produce a sympathetic skin response. Two trials were recorded per limb to check waveform reproducibility (10 s sweep, amplifier 100 mV/division, low/high pass filter settings 0.5/100 Hz). The onset latency and peak-to-peak amplitude of the response waveform with the largest amplitude was measured.

2.2.5 Quantitative sensory testing (QST)

QST is a tool that assesses the somatosensory phenotype of individuals in a standardized manner and was performed according to the protocol of the German research network of

neuropathic pain (DFNS) (Rolke et al., 2006). I underwent formal training in conducting the DFNS QST protocol at Mannheim University, Germany.

2.2.5.1 **Materials used for performance of QST**

Thermal detection and pain thresholds and thermal sensory limen (including paradoxical heat sensations) were measured using a Modular Sensory Analyzer (by Somedic, Sweden). Mechanical detection thresholds were determined using graduated von Frey hairs (Optihair2 by MARSTOCKnervetest®, Manheim, Germany). Mechanical pain thresholds and wind-up ratio were determined using graduated weighted metal rods ("The Pin-Prick", MRC Systems GmbH, Germany). Pressure pain thresholds were measured with a pressure algometer (FDN200, Wagner Instruments, USA). A 128Hz Rydel-Seiffer tuning fork was used to determine vibration detection thresholds. Mechanical pain sensitivity and dynamic mechanical allodynia were measured using the weighted metal rods, a cotton wool wisp, a Q-tip fixed to an elastic strip and a soft brush supplied with "the pin-prick" set referred to above.

2.2.5.2 **Performance of the QST protocol**

Participants were familiarized with the testing procedure on the dorsum of the forearm before all parameters were measured over the dorsum of the hand and the dorsum of the foot (S1 dermatome). Pressure pain thresholds were recorded over the thenar eminence and the arch of the foot. Vibration detection thresholds were tested over the ulnar styloid process and the medial malleolus. The QST protocol was performed unilaterally (right hand and right foot).

Thermal detection thresholds were performed first, followed by thermal pain thresholds. The modular sensory analyzer software was set to a baseline thermode temperature of 32°C with a ramp slope of 1°C/s for warming and cooling. An interstimulus interval of 5 seconds was set for thermal detection testing and 10 seconds for thermal pain testing. Participants were asked to press a hand held stop button at a) the moment they first perceived a cooling sensation as the temperature was lowered, b) the moment they perceived a warming sensation as the temperature was raised, c) the moment the cooling sensation changed its quality to an additional sensation of "burning", "stinging", "drilling", or "aching" and d) the moment the warming sensation changed its quality to an additional sensation of "burning", "stinging", "drilling", or "aching". Each of these were repeated three times and the temperature at which the stop button was pressed, recorded.

Mechanical detection threshold testing used von Frey hairs of 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512mN. Starting with 16mN, participants were asked to say "yes" if they were able to perceive a touch sensation on the area tested. If the participant could detect the stimulus the next lower intensity stimulus was given until the participant did not report detecting the stimulus. Then the next highest stimulus, and progressively higher stimuli were delivered until the participant responded "yes". This ascending-descending technique was repeated until a total of five sub- and five suprathreshold values were obtained.

Mechanical pain thresholds were determined by the same ascending-descending technique, but using weighted metal rods (8, 16, 32, 64, 128, 256 and 512mN). Starting at 8mN participants were asked to rate the stimulus as "blunt" or "pricking". As soon as the first "pricking" stimulus was perceived, the direction of testing was reversed, until the stimulus

was reported as “blunt” and then reversed again. In all five sub- and five suprathreshold values were obtained.

Mechanical pain sensitivity and dynamic mechanical allodynia were determined by applying the weighted rods, cotton wool, Q-tip or brush in a pre-determined, balanced order. Participants were asked to rate each of the stimuli on a numerical rating scale from 0-100.

Wind up ratio was assessed by delivering a 256mN pinprick once, and then repeatedly 10 times at a rate of 1 per second over a 1cm area. The participant was asked to rate the single stimulus and then to rate the perceived average of the 10 stimuli, on a numerical rating scale from 0-100. This was repeated three times and the ratio was calculated as the mean of the train: the mean of the single stimuli.

Vibration detection thresholds were measured by asking the participant to indicate when as soon as no vibration was discernible by saying “now”. A reading from 0-8 was recorded, based on the location of the “wandering” tip of a triangle attached to the tuning fork, moved by means of the vibration. The arithmetic mean of three measurements was used to determine detection threshold.

Finally, pressure pain threshold was measured by applying perpendicular downward force with the pressure algometer over the thenar eminence or abductor hallucis muscle. Force was increased at a rate of approximately 50kPa/s. Participants were asked to say “now” as soon as the sensation of pressure changed towards an additional quality of “burning”, “stinging”, “drilling” or “aching”. The force exerted at the time the participants said “now” was recorded on three consecutive occasions.

QST data were entered into the data analysis system, Equista, provided by the DFNS. Equista transformed the raw QST data into z-scores thus normalizing for age, sex, and the body location of testing (Magerl et al., 2010; Rolke et al., 2006). A z-score of zero is equal to the mean of the population. A score of greater or less than two standard deviations from the mean indicates gain of function or loss of function, respectively.

2.2.6 Grading of pain aetiology

Neuropathic pain is defined as 'pain arising as a direct consequence of a lesion or disease affecting the somatosensory system'. To determine whether the chronic pain caused by NFCI is neuropathic in nature, the grading system conceived by the Neuropathic Pain Special Interest Group (NeuPSIG) of the International Association for the Study of Pain (IASP) was used (Finnerup et al., 2016). Each study participant's pain was assessed using these published criteria as below. Possible neuropathic pain must fulfil criteria 1 and 2; probable neuropathic pain must fulfil criteria 1, 2 and 3; and definite neuropathic pain must fulfil all four criteria. (1) A history suggestive of a relevant lesion or disease affecting the peripheral or central somatosensory system—a history of neuropathy symptoms including decreased sensation, positive sensory symptoms e.g. burning, aching pain mainly in the distal areas of the upper and/or lower extremities temporally related to an episode of cold exposure. (2) Pain with a neuroanatomically plausible distribution i.e. pain symmetrically distributed in the extremities—completion of body map and clinical history. (3) Demonstration of sensory signs in the same neuroanatomically plausible distribution—sensory loss in the limb extremities. (4) Demonstration of the relevant lesion or disease by at least one confirmatory test—abnormality on nerve conduction tests, IENFD or QST.

2.2.7 Statistics

SPSS Statistics Version 21 (IBM) and GraphPad Prism were used for statistical analysis. QST z-score data were expressed as mean (95% CI). All other data were tested for normality with the D'Agostino-Pearson normality test and by visual inspection of their distribution. Data normally distributed are reported as mean (95% CI), and data not normally distributed are reported as median with interquartile range (IQR). Spearman correlation analyses were performed to explore associations between pain severity, sensory sum score (as a measure of clinical severity), time from injury to assessment, IENFD and QST findings. Significance was set at $P<0.05$.

2.3 Results

2.3.1 Study Participant demographics

2.3.1.1 NFCI participants

80 participants with chronic symptoms following NFCI were recruited for assessment from a total of 112 referrals (Figure 2.3). The participants' mean age was 32.5 (31.02-33.69) years and 75 (93.8%) of the participants were men. Most participants were originally from Africa or the Caribbean (71/80, 88.8%) (Figure 2.4).

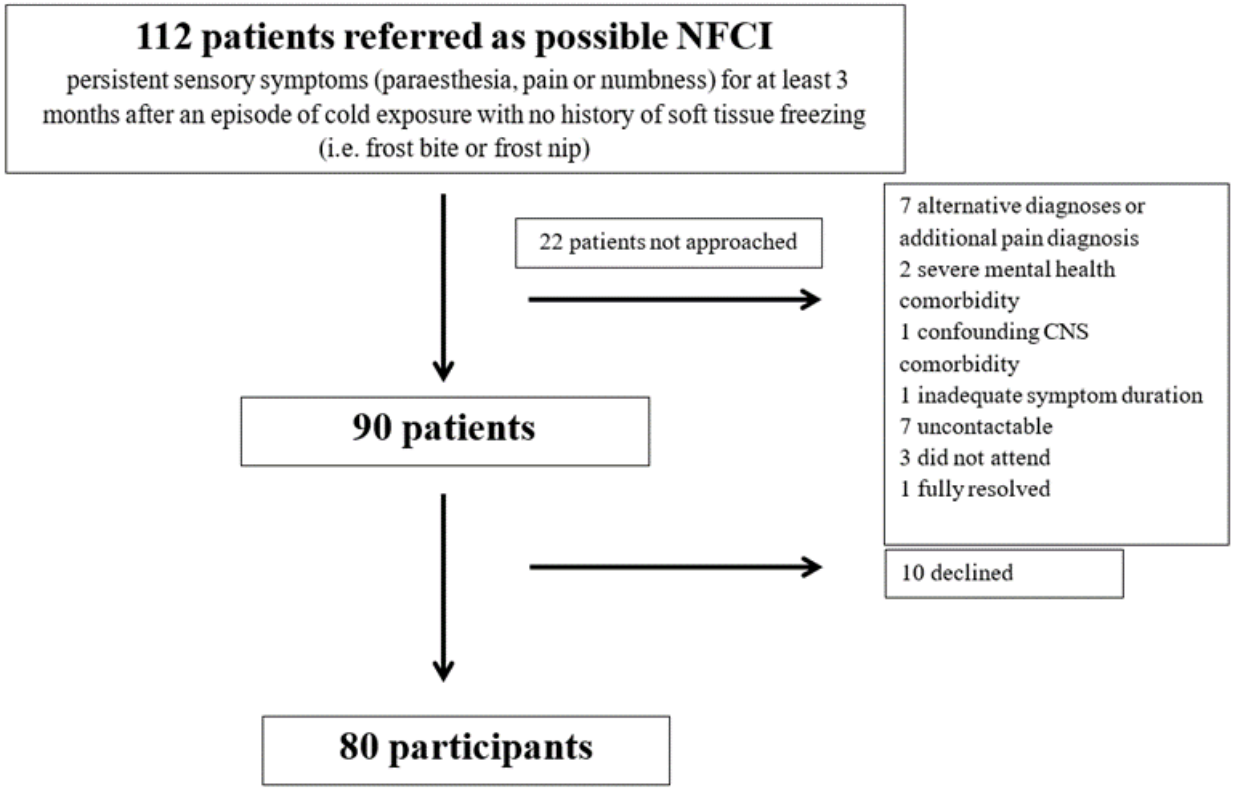


Figure 2.3 Flow chart of participant recruitment

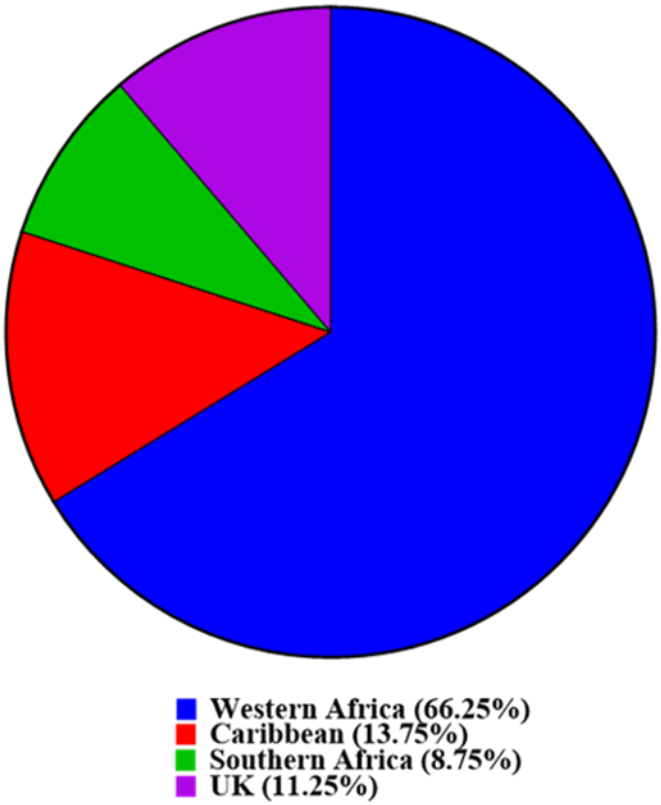


Figure 2.4 Pie chart showing region of birth of participants

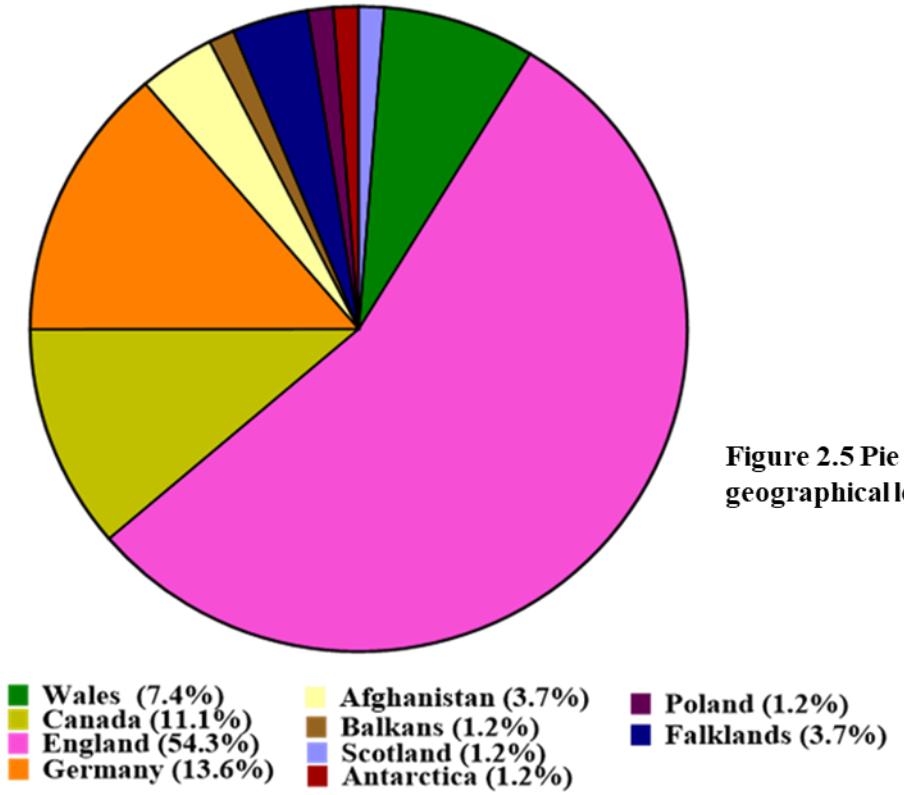


Figure 2.5 Pie chart showing geographical location of injuries

2.3.1.2 **Cold exposed control participants**

15 cold exposed control participants were recruited at Catterick Garrison, North Yorkshire. Mean age was 29.8 (27.5-32.1) years, 14 were men (93.3%), 2 from Ghana, 1 from Kenya, 1 of Nepalese heritage, 2 with one Caribbean parent and one white British parent and 9 white British. The mean length of service was 8.1 (6.2-9.9) years.

2.3.2 Narrative of Injurious Event

All apart from one of the participants suffered their injury while serving in the UK military. The civilian casualty was a volunteer on the British Antarctic Survey. The onset of symptoms was clearly related to a period of cold exposure. A specific military activity such as a cold weather exercise or tour of duty in a cold climate was cited in 97.6% of cases (Table 2.1). The majority had been exposed to cold environments on multiple occasions without ill effect before their symptoms became persistent. Similarly, most were re-exposed to a cold environment after their symptoms became persistent. The main reason for re-exposure centred on the operational/training imperatives of serving in the military. Over half of the index exposures were in England (54.8%), however injurious cold exposures occurred throughout the world (Figure 2.5). The median time from index exposure to time of study enrolment was 3.58 (2.73-6.19) years.

Index exposures	Number (%)
Cold weather	80 (100)
Wet environment	64 (80)
Duration of exposure	
< 1 hour	5 (6)
<24 hours	23 (29)
<1 week	31 (39)
1-2 weeks	2 (2.5)
2-3 weeks	4 (5)
Uncertain	15 (19)
Sleeping outdoors	53 (66.3)
Previous exposure(s)	55 (69)
Subsequent exposure(s)	60 (75)
Hands affected	75 (94)
Feet affected	79 (99)

Table 2.2 Summary of exposure histories, based on participant recollection

2.3.3 Distribution and quality of symptoms, impact of symptoms on quality of life and analgesic use

Only one participant did not experience symptoms in the feet and 75 participants (94%) reported symptoms in the hands. Pain was bilateral and symmetrical in all cases (Figure 2.6)

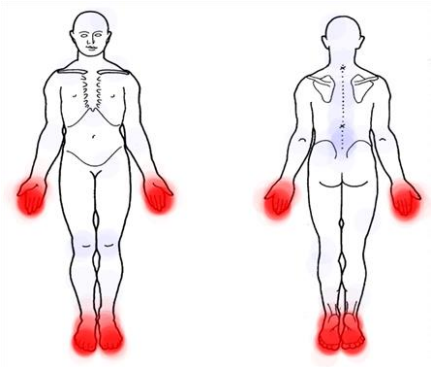


Figure 2.6 Composite heat map depicting the location of pain. Neuropathic (in red) and non-neuropathic pain (blue) reported by study participants. Study participants completed a body map highlighting the distribution of any pain experienced as part of the BPI questionnaire

In addition to pain, 98% of participants experienced numbness, 92.5% tingling and 90% painful cold. These data are shown in Figure 2.7.

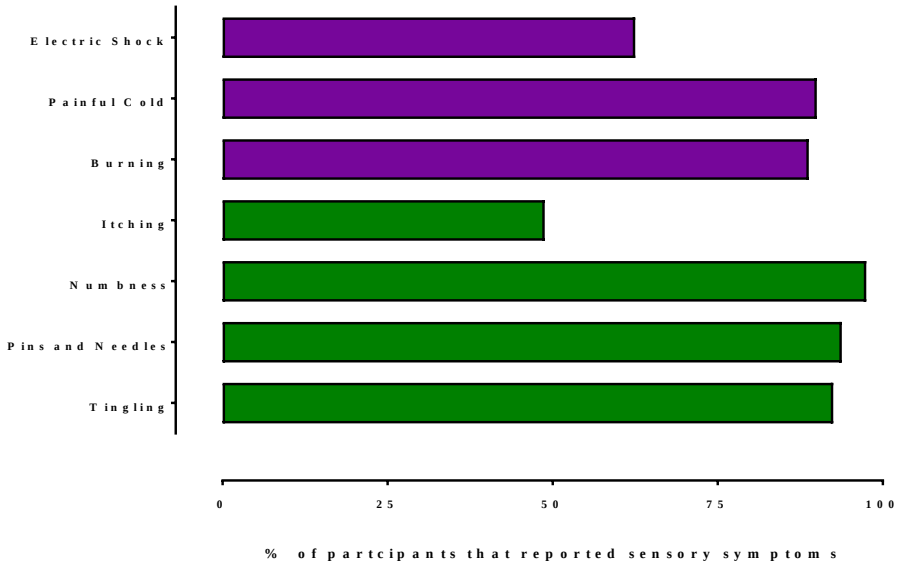


Figure 2.7 Histogram illustrating the frequency of different components from the DN4 questionnaire. The bars in purple relate to pain qualities. The bars in green indicate associated sensory phenomena.

The DN4 is a screening tool for neuropathic pain that consists of ten components. Seven items relate to pain descriptors (burning, painful cold, electric shocks) and associated abnormal sensations (tingling, pins and needles, numbness, itching). The other three items relate to a brief bedside neurological examination in the painful area (touch hypoaesthesia, pinprick hypoaesthesia, tactile dynamic allodynia). For scoring, one is given to each positive and zero to each negative item and the total score range zero to ten. A score of four or higher indicates that pain is likely to be neuropathic (Bouhassira et al., 2005). All participants had a score of greater than 4.

Many participants reported subjective impairment in temperature sensitivity in the hands or feet (55/80, 69%), resulting in a need to test water temperature with a more proximal body part and consequences such as requiring supervision when bathing children. Some participants reported causing distress to their children through running baths or showers too hot.

The severity of the pain was assessed in the Brief Pain Inventory questionnaire, in which subjects rate their pain severity on a 0-10 NRS at its worst, least, average and "right now" based on the past 24 hours experience. The severity score is calculated as the mean value of these four ratings. The median severity score was 6 (4.63 – 8). The median score on the 7-day pain diary was 7.29 (IQR 5.5-8.39).

At the time of assessment 56 participants (70%) were taking analgesia of any class. Thirteen (16%) had never taken any regular analgesia. The most commonly prescribed neuropathic pain agent prescribed at the time of assessment was amitriptyline in 25 (31.3%) participants, although another 36 (45%) had previously tried this medication. The next most commonly prescribed was pregabalin, in 19 (23.8%) individuals, trialled previously in 9 (11.3%). Gabapentin was a current medication in 9 (11.3%) and had previously been tried in 9 (11.3%). Duloxetine was a current medication in 3 (3.75%) and had previously been tried by 5 (6.25%). Nortriptyline was a current medication for 3 (3.75%) and had previously been tried by 5 (6.25%). Lidocaine patches were being used by one participant (1.25%) at the time of inclusion and had previously been used by three participants (3.75%).

The SF-36 questionnaire assesses health related quality of life (Ware & Sherbourne, 1992). This brief, yet comprehensive tool is validated for use in non-neuropathic pain states and is recommended for use in neuropathic pain research by NeuPSIG (Haanpää et al., 2011). In all the 8 domains assessed by this tool, participants with chronic pain were significantly more impaired (had lower scores) than healthy participants. This was the case for domains contributing predominantly to mental health (mental health, role emotional, social functioning), as well as those contributing predominantly to physical health (physical functioning, role physical and bodily pain) (Table 2.3).

In addition to the severity component, the Brief Pain Inventory also has an interference component, which is recommended by NeuPSIG for assessment of disability attributable to neuropathic pain. Participants rated the level of interference due to pain in 7 components, on a scale of 0 (no interference) to 10 (complete interference). The median scores (IQR) were as follows: Activity 8 (6-9), Mood 8 (6-9), walking ability 7 (3-8), normal work 8 (5-9), relations with others 7 (3-8), sleep 8 (5-10), enjoyment of life 8 (6-10). An overall pain interference score was calculated for each patient by calculating the mean score for the 7 components. The median overall pain interference score was 7.14 (5.57-8.57). These data are displayed in Table 2.3.

Sleep was severely impaired in the participants as reflected in high scores on both the Insomnia Severity Index (21 (IQR 19-26) compared to controls median score 2 (IQR 0-5)), as well as the sleep component of the Brief Pain Inventory interference score (median 8/10 (IQR 5-9)). A high proportion of respondents to this questionnaire (42 of 51, 82.4%) are

classified as having ‘clinical insomnia’ compared to 0/15 asymptomatic cold exposed participants.

The median Pain Catastrophising Scale Score was 43 (IQR 35-48), with high scores seen in the three components of helplessness, magnification and rumination. A score of 30, indicating clinical significance, was met or exceeded in 84% of respondents. Similarly, whilst control participants had median scores of 0 for all components of The Pain Anxiety Symptom Scale, a measure of the fear of pain (McCracken et al., 1992), the NFCI cohort scored highly on these components.

Significantly higher scores were reported for the negative components of the DAPOS tool ($p<0.0001$ for depression and anxiety) and significantly lower scores for ‘positive outlook’ ($p=0.0002$). The results are summarised in Table 2.3.

Measure	Cases - Median (IQR) (n=51)	Controls – Median (IQR) (n-15)	p
SF-36 Physical functioning	60 (25-80)	100 (100-100)	p<0.0001
SF-36 Role-physical	0 (0-25)	100 (100-100)	p<0.0001
SF-36 Bodily pain	22 (12-32)	100 (84-100)	p<0.0001
SF-36 General health	40 (25-61)	87 (72-97)	p<0.0001
SF-36 Vitality	40 (25-50)	72.5 (53.75-85)	p<0.0001
SF-36 Social functioning	25 (12.5-37.5)	100 (62.5-100)	p<0.0001
SF-36 Role-emotional	0 (0-33.3)	100 (100-100)	p<0.0001
SF-36 Mental health	48 (28-64)	84 (56-92)	p<0.0001
ISI	21 (19-26)	2 (0-5)	p<0.0001
Clinical insomnia	42/51 (82.4%)	0/15 (0%)	p<0.0001
BPI Interference	7.21 (5.57 – 8.64)	NA	
Activity	8 (6-9)	NA	
Mood	7.5 (4.75 – 8)	NA	
Walking ability	7 (3-9)	NA	
Normal work	8 (5-10)	NA	
Relations with others	6.5 (3-9)	NA	
Sleep	8 (5-9)	NA	
Enjoyment of life	8 (6-10)	NA	
DAPOS			
Depression	15.5 (9-20.25)	5 (5-7)	p<0.0001
Anxiety	9 (7-13)	3 (3-7)	p<0.0001
Positive outlook	6 (4-8)	11 (9-14	p=0.0002
PCS			
Helplessness	19 (15.25-21)	0 (0-0)	p<0.0001
Magnification	10 (6.25-12)	0 (0-0.25)	p<0.0001
Rumination	15 (13-16)	0 (0-0.5)	p<0.0001
Total	43 (35-48)	0 (0-1.25)	p<0.0001
PASS			
Cognitive	19.5 (14-23)	0 (0-1)	p<0.0001
Escape avoidance	18 (12.75-21)	0 (0-3)	p<0.0001
Fear	19 (14-23)	0 (0-0)	p<0.0001
Physiological anxiety	13 (6-18)	0 (0-0)	p<0.0001
Total	67.5 (48-78.75)	0 (0-6)	p<0.0001

Table 2.3 Measures of pain severity and psychosocial factors in participants with chronic pain compared to a group of servicemen exposed to similar cold conditions but without NFCI. SF-36, Short From 36 of the MOS Outcomes Study; BPI, Brief Pain Inventory; DAPOS; Depression, Anxiety, Positive Outlook Scale; PCS, Pain Catastrophising Scale; PASS, Pain Anxiety Symptom Scale 20). Mann-Whitney U Test

2.3.4 Structured neurological examination, nerve conduction studies and intraepidermal nerve fibre densities

One participant had mild weakness of extensor hallucis longus on one side (4+/5). This participant had normal bilateral sural and common peroneal nerve conduction studies. Otherwise no participant had weakness in any of the tested muscles. Reinforcement was required to elicit ankle tendon reflexes in one participant. Ankle reflexes could not be elicited in four participants despite reinforcement.

The sensory sumscore is calculated based on how far proximally deficits are present in pinprick, light touch, vibration sense and joint position sense (as described in the methods section). The sensory sumscore increases as deficits become more proximal (i.e. higher scores indicate worse sensory function). The median sumscore in this cohort was 10 (IQR 6.25-12.0), with abnormalities present across the different modalities. The most frequently impaired modality in the upper and lower limbs was pinprick sensation, with impairments observed in 85% and 92.3%, respectively. Only one participant had impairment of pinprick sensation proximal to the wrist. Three participants had bilateral impairment proximal to the ankle. One participant had unilateral impairment proximal to the ankle. After pinprick sensation the next most frequently impaired modality in upper and lower limbs was light touch (71.3% and 80%), then joint position sense (27.5% and 45%) and finally vibration sense (18.8% and 30%).

Number of participants	80
Bilateral impairments on sensory examination of the hands	
- Pinprick	68 (85.0%)
- Light touch	57 (71.3%)
- Proprioception	22 (27.5%)
- Vibration	15 (18.8%)
Bilateral impairments on sensory examination of the feet	
- Pinprick	74 (92.3%)
- Light touch	64 (80.0%)
- Proprioception	36 (45.0%)
- Vibration	24 (30.0%)
Sensory Sum score	10 (6.25-12.0)
Absent Ankle Jerks	4 (5.0%)
IENFD (fibres/mm)	3.9 (3.5-4.3)
Nerve conduction studies	
Sural nerve	
- Amplitude (μV)	15.6 (11.4-22.0)
- Conduction Velocity (m/s)	47.1 (43.8-52.1)
Peroneal nerve (motor)	
- Amplitude (mV) (ankle-EDB)	9.1 (6.9-12.9)
- Velocity (m/s) (Fibular head – ankle)	49.5 (45.9-52.6)
Sympathetic skin response (mV)	1.3 (0.6-2.8)

Table 2.4 Summary of pertinent clinical examination findings, electrophysiological recordings and IENFD. Nerve conduction study data were performed for 60 (75.0%) study participants in the lower limbs. The nerve conduction studies and sympathetic skin response values are expressed as median (IQ). IENFD results were obtained for 78 participants (97.5%) and expressed as mean (95%CI)

2.3.5 Nerve Conduction Studies

Sixty participants (75%) had nerve conduction studies. Sural amplitudes and velocities were normal in all participants tested. One participant had unrecordable responses from the extensor digitorum brevis muscles on both sides, with clinical absence of the muscle, bilaterally. This was interpreted as a congenital anomaly as he had no weakness and could not recall any history compatible with an acquired injury to the peroneal nerve (except for his NFCI, following which he never had subjective weakness). No significant difference was seen in the mean 7 day pain diary score between those who had nerve conduction studies and those who did not (p=0.089, Mann-Whitney test). Those who underwent nerve conduction studies did have significantly more impaired sensory examination than those who did not, with a median score of 7 in those who did not undergo NCS, and 10 in those who did undergo NCS (0.0467, Mann-Whitney test), yet the values obtained at the sites tested were within normal reference limits.

2.3.6 Skin Biopsy

Seventy eight participants (97.5%) underwent skin biopsy 10cm proximal to the ankle joint. The mean intra-epidermal nerve fibre count was 3.9 fibres per millimetre (95% CI 3.5-4.3). The values for 60 participants (75%) were below the 0.05 quantile of published reference values (Lauria et al., 2010) for their age and sex. Three values were *at* the 0.05 quantile value, and the remaining 15 were above. None of the IENFD values reached the normative mean value for corresponding age and sex.

There are no published normative IENFD values for African or Caribbean individuals. Age and sex matched data for healthy African Americans were provided by The Cutaneous Nerve

Laboratory, Johns Hopkins School of Medicine. The IENFDs for these individuals were significantly higher than for the NFCI patient cohort (Figure 2.8A; 10.8 fibres per millimetre; 95% CI 8.9-12.6). Similarly the IENFD for a group of soldiers exposed to similar cold conditions but without a history of NFCI, were significantly higher (12.2 fibres per millimetre; 95% CI 10.2-14.1) (Figure 2.8B).

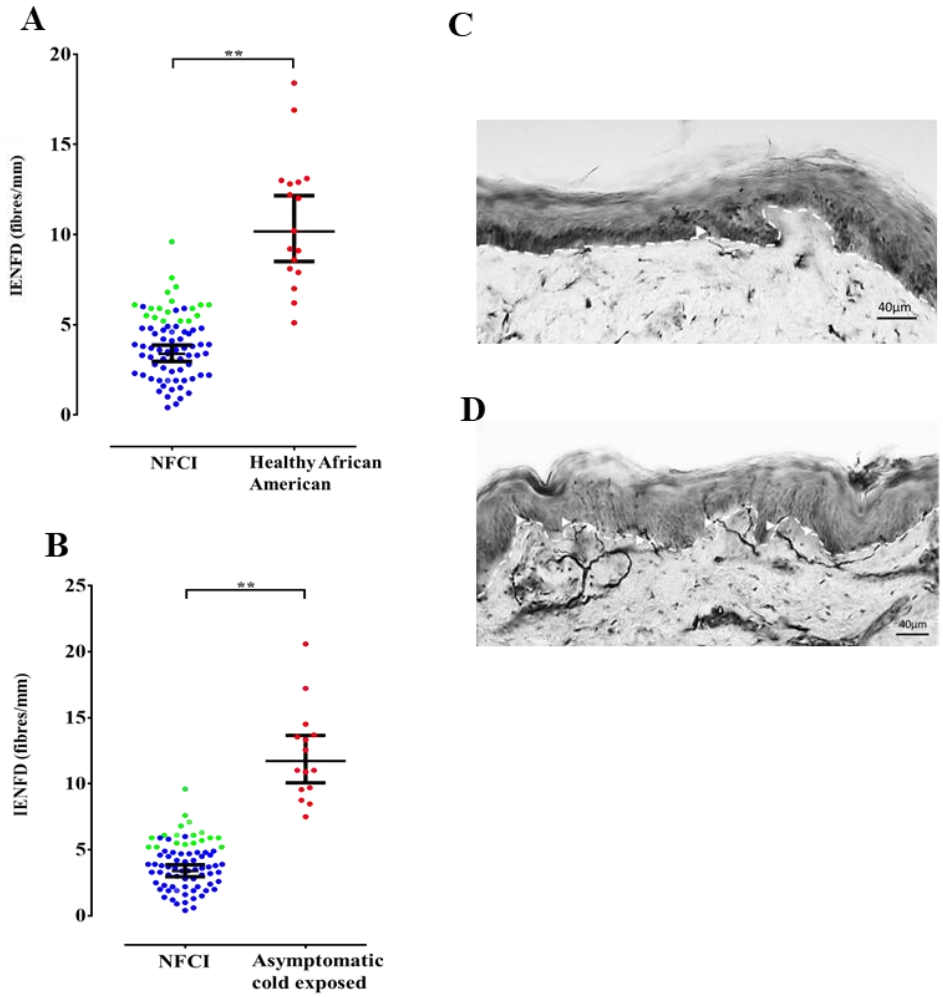
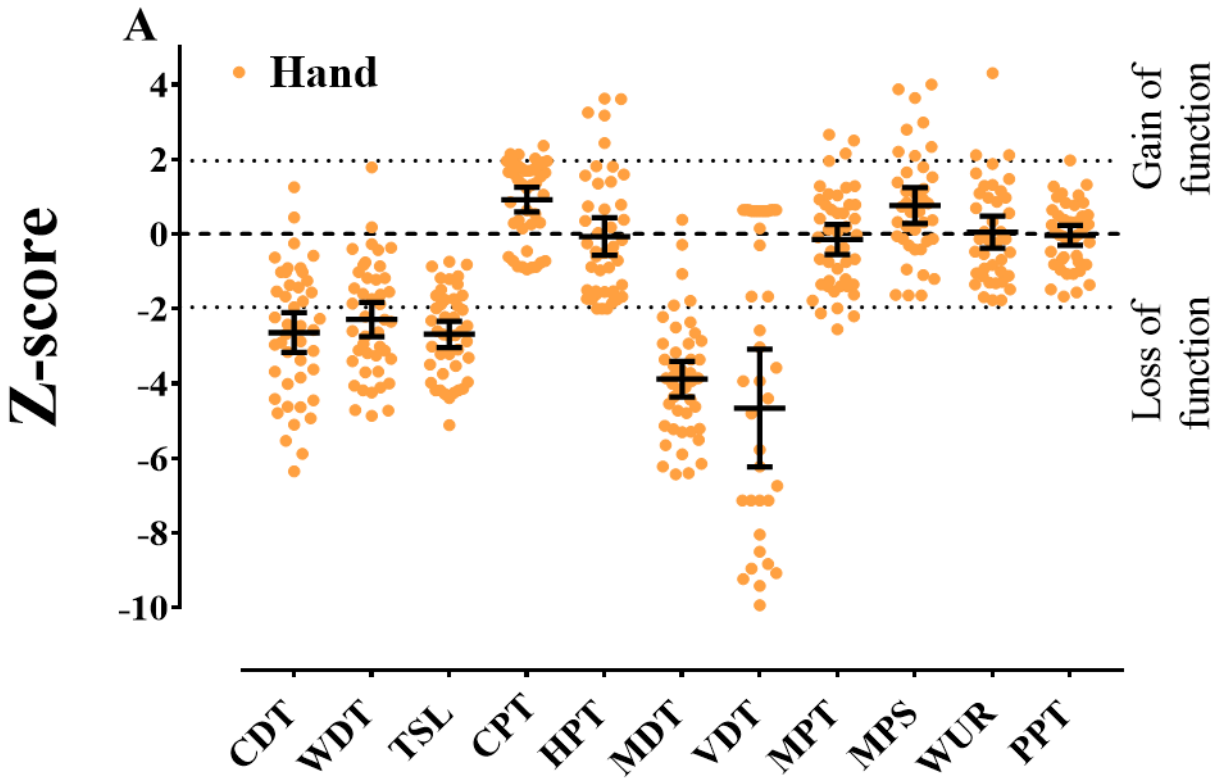


Figure 2.8 NFCI participants had significantly lower intraepidermal nerve fibre densities than control participnats Blue circles represent values below the 0.05 quantile reference value for age/gender. Green circles represent values at or above the 0.05 quantile reference value. Red circles in **A)** represent values for healthy African American participants and in **B)** for age matched individuals exposed to similar cold conditions to cases but without symptoms of NFCI. ** P<0.0001. There is no significant difference between healthy African Americans and asymptomatic cold exposed (p=0.27) **C)** Representative image of skin biopsy from NFCI participant. **D)** Representative image of skin biopsy from healthy control

2.3.7 Quantitative sensory testing

Forty four patients underwent quantitative sensory testing. In the hand mean z scores for the whole cohort were below 1.96 standard deviations of the mean normative DFNS dataset in several thermal and mechanical modalities: cold and warm detection thresholds (CDT and WDT, respectively), thermal sensory limen (TSL) and mechanical and vibration detection thresholds (MDT and VDT, respectively). The mean values for thermal pain thresholds, mechanical pain threshold and pain sensitivity, as well as wind up ratio and pressure pain threshold, fell within the normative range of the DFNS dataset.



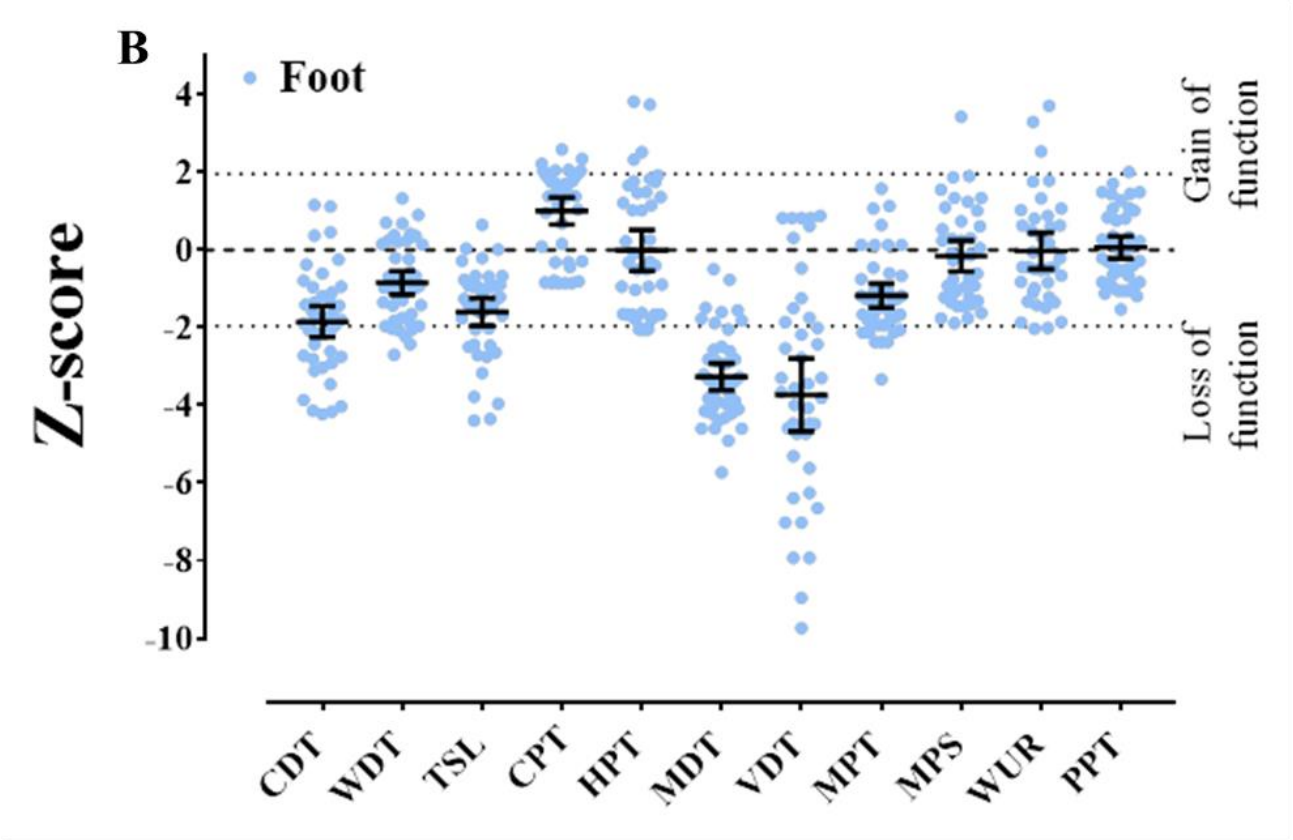


Figure 2.9 Scatter plot and mean (95% CI) for QST parameters in the A) hand and B) foot

By comparing participant data to normative data from the DFNS a z-score can be generated, which indicates the number of standard deviations from the mean control population. A z-score of zero is equal to the mean of the population; a score of greater, or less, than two standard deviations (1.96) from the mean indicates gain, or loss, of function respectively. In the hand mean z-scores were less than -1.96 for CDT, WDT, TSL, MDT and VDT. In the foot mean z-scores were below -1.96 only for MDT and VDT. Reductions were seen for CDT, WDT and TSL, although these were not to below -1.96.

CDT, cold detection threshold; CPT, cold pain threshold; DMA, dynamic mechanical allodynia; HPT, heat pain threshold; MDT, mechanical detection threshold; MPS, mechanical pain sensitivity; MPT, mechanical pain threshold; PHS, paradoxical heat sensation; PPT, pressure pain threshold; TSL, thermal sensory limen; VDT, vibration detection threshold; WDT, warm detection threshold; WUR, wind-up ratio

Foot					Hand			
Modality	Actual Value	Z Score	% with gain of function	% with loss of function	Actual Value	Z score	% with gain of function	% with loss of function
CDT	18.22 (15.74-25.71)	-1.85 (-2.52 to -1.41)	0	43	24.09 (22.17 – 26.00)	-2.64 (-3.17 to -2.11)	0	57
WDT	36.43 (35.03 – 37.82)	-0.85 (-1.15 to -0.54)	0	14	38.06 (36.29 – 39.81)	-2.29 (-2.75 to -1.83)	0	55
TSL	18.92 (15.76 – 22.08)	-1.60 (-1.95 to -1.24)	0	30	15.65 (12.58 – 18.71)	-2.68 (-3.03 to -2.33)	0	67
CPT	19.65 (16.69 – 22.61)	1.00 (0.66 – 1.35)	18	0	19.92 (17.03 – 22.81)	0.92 (0.59 – 1.25)	7	0
HPT	45.51 (44.20 – 46.83)	-0.01 (-0.54 – 0.52)	9	9	44.89 (43.41 – 46.37)	-0.07 (-0.57 – 0.43)	7	11
MDT	180.6 (122.10 –239.10)	-3.27 (-3.61 to -2.92)	0	82	56.77 (35.75 – 77.79)	-3.88 (-4.36 to -3.41)	0	89
VDT	4.99 (4.38 – 5.60)	-3.74 (-4.68 to -2.80)	0	70	6.00 (5.40 – 6.59)	-4.66 (-6.24 to -3.08)	0	62
MPT	349.60 (282.50 – 416.70)	-1.18 (-1.49 to -0.86)	0	18	186.0 (127.70 –244.30)	-0.15 (-0.55 – 0.26)	11	7
MPS	1.91 (-0.18 – 3.64)	-0.16 (-0.56 – 0.24)	2	0	4.33 (1.68 – 6.99)	0.76 (0.28 – 1.24)	26	0
WUR	3.76 (2.27 – 5.26)	-0.03 (-0.49 – 0.28)	7	5	2.58 (1.90 – 3.27)	0.05 (-0.38 – 0.48)	8	0
PPT	630.60 (558.90 – 702.20)	0.07 (-0.22 – 0.35)	2	0	503.0 (452.60 – 553.40)	-0.04 (-0.30 – 0.23)	2	0

Table 2.5 QST values for the foot (left) and hand (right). Mean values (95% CI). In the foot mean z scores were below 1.96 standard deviations of the mean normative DFNS dataset in mechanical and vibration detection. The mean z score for CDT was -1.85. The mean values for WDT, thermal pain thresholds, mechanical pain threshold and pain sensitivity, as well as wind up ratio and pressure pain threshold, fell within the normative range of the DFNS dataset. The implication of this result is that there are impairments in function of large fibres. The mean value for CDT (a small fibre mediated modality) is at the lower end of the normative range. In the hand mean z scores were below 1.96 standard deviations of the normative DFNS dataset for thermal detection in addition to mechanical and vibration detection, implying impairments in small and large fibre function.

Those values falling below 1.96 standard deviations of the normative dataset reflect losses of function in cold and warm detection, as well as mechanical and vibration detection. The implication of this result is that there are impairments in function of small fibres (subserving temperature detection) and also large fibres (subserving mechanical detection and vibration detection).

Despite the uniformly reported symptom of cold hypersensitivity, as a cohort, no significant gain of function was seen in the hand or foot for cold pain threshold. A significant correlation is seen between z score for cold detection threshold and z score for cold pain threshold i.e. the higher the cold detection threshold (loss of function for cold detection), the higher the cold pain threshold (loss of function for cold pain perception) (Pearson r=0.6662, p<0.0001) (Figure 2.10).

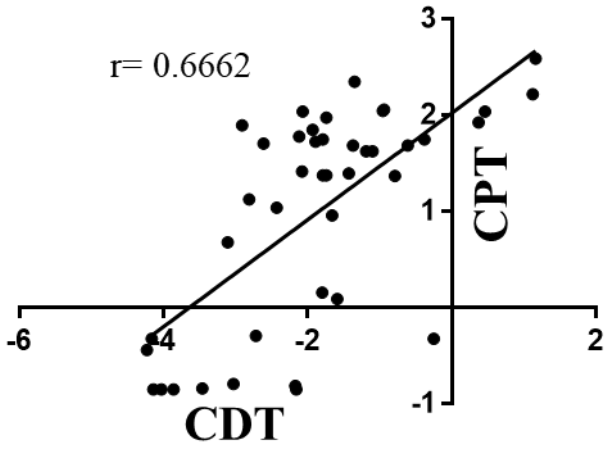


Figure 2.10 Correlation between cold detection threshold and cold pain threshold.

Fourteen participants (31.8%) perceived a cooling stimulus as hot in the foot during the thermal sensory limen protocol. Four of these fourteen reported the same phenomenon in the hand (9.0%). Another 5 participants (11.4%) reported this in the hand, only. Dynamic mechanical allodynia was seen in only one participant (2.3%).

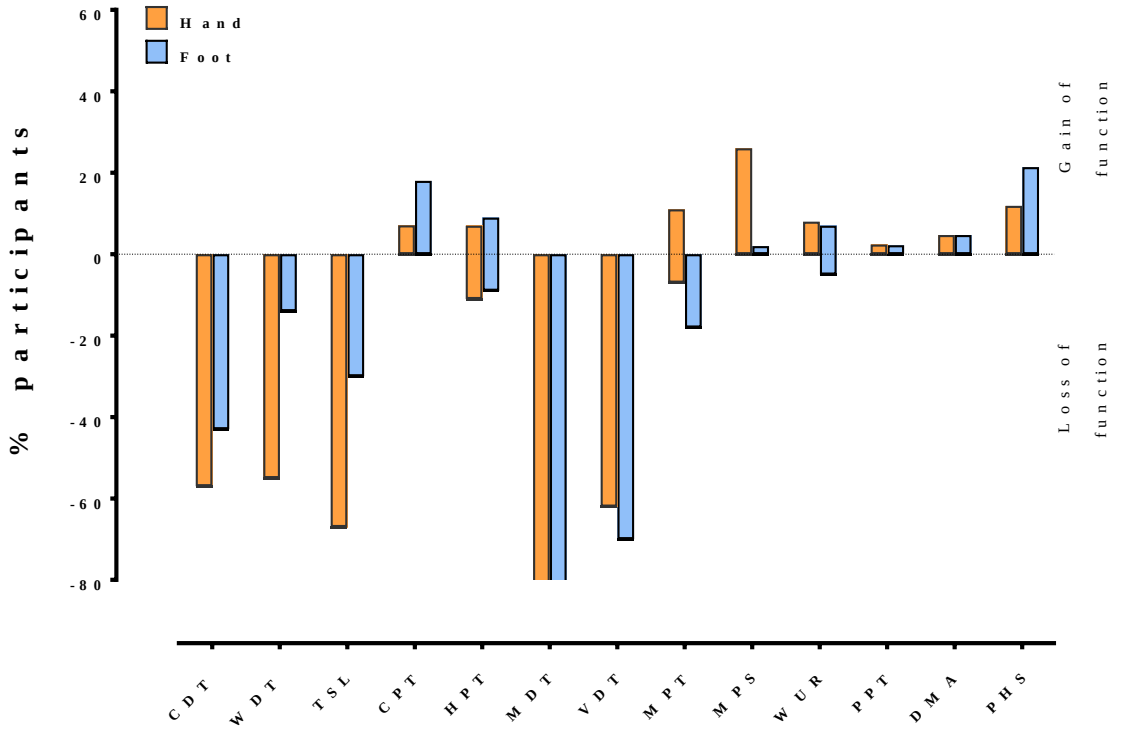


Figure 2.11 Loss or gain of sensory function in the hand and foot

The y-axis shows the percentage of patients in each group with 'gain' of sensory function plotted upwards and "loss" of sensory function plotted downwards i.e. z-scores that were greater or less than two standard deviations from the mean. The overall pattern for QST parameters is similar to the mean z-scores, with the highest frequency of loss of function in the thermal and mechanical detection thresholds. Only a minority of study participants exhibited gain of function phenomena within the pain detection parameters. Paradoxical heat sensations were elicited in 48.1% of participants (3.7% in both hand and foot, 29.6% in the foot alone and 14·8% in the hand alone) and dynamic mechanical allodynia was elicited in only two participants.

CDT, cold detection threshold; CPT, cold pain threshold; DMA, dynamic mechanical allodynia; HPT, heat pain threshold; MDT, mechanical detection threshold; MPS, mechanical pain sensitivity; MPT, mechanical pain threshold; PHS, paradoxical heat sensation; PPT, pressure pain threshold; TSL, thermal sensory limen; VDT, vibration detection threshold; WDT, warm detection threshold; WUR, wind-up ratio.

2.3.8 Grading

All participants reported a history of a relevant lesion. They all described chronic pain that was temporally related to one or more prolonged cold exposures. All patients reported pain in a neuroanatomically plausible distribution and had abnormalities on sensory examination corresponding to the same distribution. The intraepidermal nerve fibre density was below the 0.05 quantile in 60 of 78 (76.9%) participants and therefore these can be said to have definite neuropathic pain. Of the 18 participants whose IENFD was at or above the 0.05 quantile, 10 were investigated by QST and all of these participants demonstrated abnormalities in at least one modality tested (Figure 2.12). If QST is classified as a confirmatory test the number of participants with definite neuropathic pain rises to 70/80 (87.5%). In the most recent iteration of the NeuPSIG grading criteria (Finnerup et al., 2016) QST is not listed as a specific diagnostic test. However in the 2011 NeuPSIG Guidelines on neuropathic pain assessment, it is stated the QST findings are considered a confirmatory diagnostic test in the neuropathic pain grading system, since QST can provide independent verification of sensory signs (Haanpää et al., 2011).

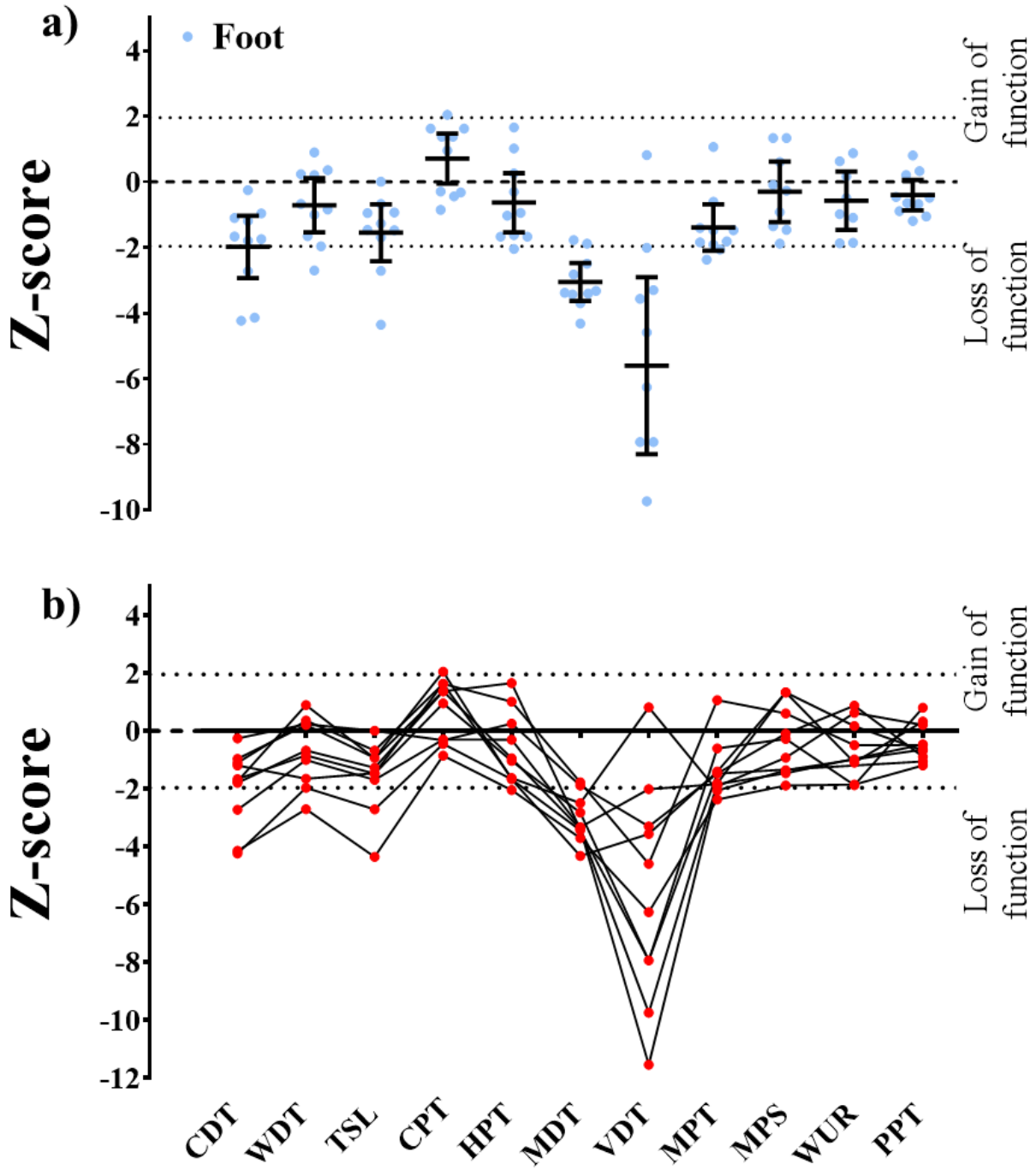


Figure 2.12 QST profiles for 10 participants with IENFD ≥ 0.05 reference quantile. a) Scatter plot and mean (95% CI) for QST parameters in the foot and **b)** Individual QST profiles

CDT, cold detection threshold; CPT, cold pain threshold; DMA, dynamic mechanical allodynia; HPT, heat pain threshold; MDT, mechanical detection threshold; MPS, mechanical pain sensitivity; MPT, mechanical pain threshold; PHS, paradoxical heat sensation; PPT, pressure pain threshold; TSL, thermal sensory limen; VDT, vibration detection threshold; WDT, warm detection threshold; WUR, wind-up ratio

2.3.9 Relationship between symptom severity and neuropathy severity, duration of injury, IENFD and QST measures

There is a statistically significant correlation between pain severity and neuropathy severity (as measured by BPI Severity score versus sensory sumscore) (r=0.42, p=0.0001; Spearman Correlation). There is a significant inverse relationship between duration of symptoms since injury and severity of neuropathy, as measured by sensory sumscore (r= -0.34, p=0.002; Spearman Correlation) (Figure 2.13).

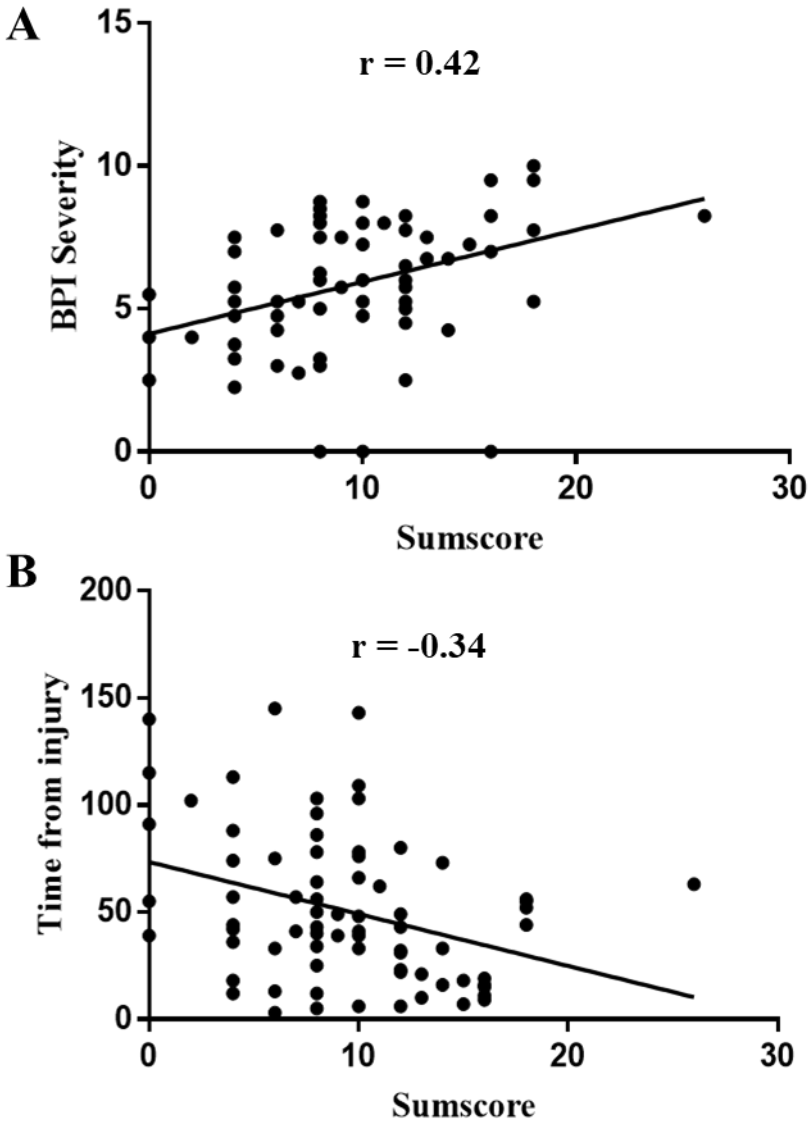


Figure 2.13 Correlations between A) Pain severity and examination severity and B) Chronicity of symptoms and examination severity

The sensory sumscore was significantly inversely correlated with MDT z scores for both the hand and the foot with Spearman correlation co-efficients of -0.638 and -0.455, respectively. There was also an inverse correlation between sumscore and MPT z scores for hand (r= -0.417) and foot (r= -0.410) There were no relationships between sensory sumscore and other QST measures, or with IENFD.

2.4 Discussion

Just as Paddock postulated in 1946, the data shown in this thesis demonstrate that "the terminal nerve twigs are at fault". In the chronic phase, NFCI results in a painful sensory neuropathy, characterised by both small and large fibre deficits. The chronic pain experienced in NFCI is neuropathic in nature. It is severe and disabling. Following a relevant lesion (cold exposure), pain is experienced in a glove and stocking distribution, with concordant examination findings and confirmation of a lesion on QST or skin biopsy. The data presented in this chapter pertain predominantly to young African and Caribbean men.

The pain itself has characteristics that are suggestive of neuropathic pain, as demonstrated by the results of the DN4 questionnaire, with 89% of participants reporting a burning quality and 90% painful cold. Pins and needles and numbness were reported by 93.8% and 97.5%, respectively. In line with previous studies all patients reported a worsening of symptoms upon exposure to cool/cold conditions or environments. Importantly the degree of coldness necessary to elicit symptoms would *not* have elicited such symptoms prior to the NFCI. The deleterious effects on quality of life, psychological wellbeing and sleep are reflected in the high scores seen in several questionnaires used to assess these domains.

There were marked abnormalities on QST of the hands and feet in the form of hyposensitivity relating to thermal parameters mediated by small fibres including warm detection, cool detection and heat pain, as well as hyposensitivity to stimuli activating large myelinated sensory fibres such as mechanical detection and vibration sense. Overall the pattern is consistent with sensory loss or 'deafferentation' profile (Baron et al., 2017) affecting both hands and feet.

2.4.1 Comparison to published datasets

A paper pertaining to analysis of 42 participants in this cohort was published in 2017 (Vale et al., 2017). The findings presented in this thesis reinforce the findings from that publication. Since then, an 'audit' by Anand *et al* (Anand et al., 2017) has also replicated several of the findings. Thirty soldiers with a history of persistent sensory symptoms following cold exposure were recruited, twenty of whom (67%) were African/Caribbean (cf 89% of the cohort in this thesis). The participants were recruited a mean of 27 months following injury, compared to 50 months in this cohort. Not all participants in the Anand publication reported pain, although it was present in a majority (86%), the remainder reporting "other" symptoms possibly including pins and needles, electric shock sensations and itch.

	Vale	Anand
Total Number Participants	80	30
Mean age (years)	32.5	31
Proportion African/Caribbean	89%	67%
Number of women	5 (6.25%)	1 (3.3%)
Mean time from injury (months)	50	27
Pain worsened by cold exposure	100%	86%
Pins and needles	95%	63%
Impaired pinprick in the feet	92%	67%
Impaired joint position sense in the toe	45%	17%
Impaired MDT in the foot	82%	63%
Impaired VDT in the foot	70%	40%
Impaired CDT in the foot	43%	73%
Impaired WDT in the foot	14%	83%
Abnormal CPT in the foot	18%	64%
Proportion with abnormal calf IENFD	77%	90%*
Mean IENFD (fibres/mm)	3.9 (3.5-4.3)	3.18**
Abnormal SNAPs in the feet	0%	23%***
Absent SSRs	1.3%	0%

Table 2.6 Comparison of key data between this thesis and Anand *et al* (Anand et al., 2017)

*This is the percentage abnormal relative to an "in house" control sample. The value for the sample in this thesis is relative to the normative data published by Lauria *et al* (Lauria et al., 2010). **This figure is the mean IENFD in 50uM sections for 15 participants. All skin biopsies were cut at 15uM and the IENFD reduced in all of them but the absolute number is not reported. *** The reported abnormalities are in the plantar nerves, whereas the sural nerve was recorded in this thesis.

It is interesting to compare these two datasets (Table 2.6), although caution is required due to some differences in cohort characteristics and methodological differences. The sample size is much smaller in Anand's publication and the injuries sustained by Anand's cohort are more recent. Two thirds of his cohort were African or Caribbean compared to 89% in the cohort reported here. QST data were collected and analysed according to the DFNS protocol in this work, which was not the case for Anand, who used different, unpublished criteria to define normality. Another potentially important difference is that whilst Anand used the sole of the foot for thermal parameters the dorsum of the foot was used here. Anand measured vibration using a biothesiometer at the great toe, whilst in this study a Rydel Seiffer tuning fork was used at the lateral malleolus.

Whilst there are differences in the frequencies of abnormal examination findings and QST parameters, both datasets reveal deficits in small and large fibre function and both have IENFDs below 'internal' control values as well as the widely used Lauria reference values (Lauria et al., 2010). The majority of Anand's participants had normal SNAPs in the feet, measured from the plantar nerve.

As stated above, although there are broad similarities, there are differences in the proportions of participants exhibiting various deficits, clinically, on QST and electrophysiologically. Interestingly large fibre deficits were less frequently seen in Anand's cohort, both on clinical examination and on QST. Conversely, temperature indices were more frequently reported as abnormal compared to this cohort. These discrepancies may be accounted for by the different methodologies used. Anand specified an inability to detect cooling of less than 2.4 degrees as abnormal. If this criterion is applied to the cohort studied in this thesis then the

proportion of participants with abnormal cold detection thresholds rises from 43% to 90.9%. Anand's publication does not provide any detail regarding cold pain thresholds. The normative data used by Anand are unpublished and so it is difficult to make further meaningful commentary.

2.4.2 Discrepancy between CDT and CPT, and subjective and objective cold hypersensitivity.

Although every participant reported cold hypersensitivity, a gain of function in cold pain thresholds was seen in only 18% of this cohort. Although deficiencies in cold detection were observed in the foot of 43% of those who underwent QST, this was only observed in one of the eight participants (12.5%) who displayed gain of function for CPT. It is logical that the presence of a significant correlation between z scores for CDT and CPT suggests that if participants were unable to detect innocuous cold they were also less able to perceive noxious cold. However, this still does not account for the discrepancy between the reported symptom of cold hypersensitivity and the relatively low occurrence of gain of function for CPT. This may be accounted for by the fact that in the DFNS healthy control cohort (the reference group used for this work), the range of temperatures at which cold was perceived as painful was very large compared to the range for cold detection. Consequently the 95% confidence intervals for thermal pain reference data are wide and reach the limits of the measurement range **(Rolke et al., 2006).**

Namer *et al.* investigated patients diagnosed with non freezing cold injury during their service in the Norwegian army (Namer et al., 2008). They exhibited markedly reduced cold pain thresholds (mean temperature threshold for pain elicitation 24.2°C compared to 14.8°C

for the control group) compared to an internal control group. Interestingly, the authors comment that their NFCI patients differ from their neuropathy patients, insomuch as they tolerate further cooling after the cold pain threshold, whereas their neuropathy patients with cold allodynia are unable to tolerate this. In contrast to the cohort studied in this thesis, as well as in the Anand paper, Namer's patients had normal warm and cold detection thresholds (compared to internal control values). This may be accounted for by a difference in severity of the inciting injury. Details of neurological examination are not given in the publication.

The context in which the cold stimulus is given, including the 'meaning' of, and visual attention to, the stimulus, may also be important (Moseley & Arntz, 2007; Arntz & Claassens, 2004). The delivery of a localised cold stimulus to part of the hand or foot via a 50mm x 25mm thermode, in a controlled and safe laboratory environment, is very different from exposure to a generally low ambient temperature with abundant situational cues.

Another potential explanation is that whilst there may have been deafferation of a population of nociceptors as a result of NFCI, there may have been *sensitisation* of a population of unaffected nociceptors as a result. This would translate to complex outcomes in terms of final pain perception, especially in a heterogeneous population. Deficits may be masked by hypersensitivity in adjacent areas (Jensen & Finnerup, 2014).

2.4.3 Discrepancy between large fibre deficits and normal nerve conduction studies

It is a paradox that large fibre abnormalities are present without any impairment in distal sensory or motor neurophysiological indices. Although not all participants had these tests performed there was no significant difference in neuropathy severity (as measured by sensory sumscore) between those who did and those who did not have these tests (p=0.16 unpaired t test).

One possible explanation would be that large fibre injury is sufficiently distal that it is not detected by sural (the most distal site examined in this cohort) nerve conduction studies. There could be injury of neuronal and/or non-neuronal components of mechanosensory end organs (including Merkel Cells, Meissner corpuscles, Ruffini endings and Pacinian Corpuscles) that certainly wouldn't be detected by nerve conduction studies. The anatomy of these structures is both highly ordered and complex, adapted to the role that these structures must perform (Zimmerman et al., 2014). Inevitably**,** given this complexity, there are many potential mechanisms for disruption of normal function. These structures are found primarily in glabrous skin and deep in hairy skin (Myers et al., 2013), the sampling of which from the participants in this study was precluded due to ethical constraints. It was not permitted to take skin from the finger or from more distal than the ankle joint.

2.4.4 Intraepidermal nerve fibre densities

In this cohort 76.9% of participants had IENFD below the 0.05 quantile reference value for their age and sex (Lauria et al., 2010). In the context of the clinical histories and examination findings this confirmed the presence of structural injury to small sensory neurons. None of the remaining 18 participants' values met the median age and sex matched reference value.

There are no published normative data for IENFD in African or Caribbean populations. Therefore, the participants' results were compared to a group of healthy African American participants and there was a significantly reduced IENFD compared to this latter group, too. In one published American reference cohort of 98 healthy individuals, 18% are African American. The authors note that there was no significant influence of race on IENFD (McArthur et al., 1998). There is a paucity of normative data for all ethnic groups aside from white European individuals and relatively little research has examined ethnic differences. Shikuma *et al.* (Shikuma et al., 2013) measured significantly higher IENFDs in healthy Thais compared to published normative data for a healthy American cohort (McArthur et al., 1998). Similarly in a small cohort of American Chinese small fibre neuropathy patients IENFD was found to be significantly higher than for non-Chinese American small fibre neuropathy patients (Jin et al., 2018). Two control groups have been used in this study, one comprising healthy, age matched African American individuals, and another comprising age-matched, cold exposure matched individuals. The ideal control group would be one matched for age, cold exposure *and* ethnicity, but unfortunately it has not been possible to obtain significant numbers of controls meeting all these requirements.

No correlation was observed between symptom severity or the severity of examination findings, and IENFD. This is not necessarily surprising given that IENFDs were generally low and there was likely to have been a 'flooring effect'

2.4.5 Secondary gain as a potential confounder

In his cohort of soldiers injured by frostbite Blair noted "all symptoms are more severe than organic physical findings would indicate" and that "monetary compensation from the Veterans Administration may be involved" (Blair et al., 1957). Participants in this study were not asked whether or not they were engaged, or planning to engage, in litigation against the Ministry of Defence. It is recommended that QST not be undertaken in individuals "with pending litigation or compensation, particularly as it has not been possible in prior studies to distinguish those who are feigning from those who are not (Backonja et al., 2013). It is assumed that all participants enrolled in this project did so in good faith, however the possibility of conscious alteration of responses by participants cannot be fully excluded. All participants were advised prior to consenting that results would not be fed back, and that results would remain confidential. Therefore participants would have known that there was no potential secondary gain from feigning responses.

2.4.6 Psychosocial functioning in patients with chronic symptoms

The sensory neuropathy seen in this cohort results in a chronic pain syndrome. In common with all other chronic pain syndromes there is a significant psychosocial component. This has never previously been studied in NFCI. Whilst it is possible that these patients represent the more severe end of a spectrum, it seems unlikely that such psychosocial sequelae are not also present in the wider population of patients in the chronic phase of NFCI.

It was not an objective of this work to investigate the role of ethnicity in pain phenotype, and this study was not designed to meet that objective. However it is important to acknowledge that the majority of participants were African or Caribbean, and that there are

published, clinically meaningful, ethnic differences in pain severity and psychological measures intricately linked to pain experience and reporting (Forsythe et al.,2011; Fabian et al., 2011; Kim et al., 2019)

Every individual in this cohort had been discharged or downgraded (and many of these awaiting discharge) as a result of their symptoms. The fates of these participants in terms of their ongoing employment are not yet known. This has been investigated in soldiers discharged to civilian life following the WWII (Abramson et al., 1946) and The Korean War (Blair et al., 1957), albeit in long term survivors of frostbite rather than NFCI in the case of the latter. In the Abramson cohort 72% of those surveyed reported that they were unable to perform their role as well as the man working next to them. In Blair's Korean Veterans, 91/97 veterans were in employment 4 years after injury. However, more than half of these reported difficulty performing their roles, and "several" were unable to accept outdoor jobs during winter months, presumably due to an exacerbation of their symptoms.

High scores and significant differences from cold exposed control participants were seen across a variety of questionnaires validated for use in researching relevant psychosocial domains. The SF-36 questionnaire is validated for use in measurement of health related quality of life in non-neuropathic pain states and is recommended for use in neuropathic pain research by NeuPSIG (Haanpää et al., 2011). In all of the 8 domains assessed by this tool, participants with chronic pain were significantly more impaired (had lower scores) than healthy participants. Sleep, too, was severely impaired in the participants as reflected in high scores on both the Insomnia Severity Index (median score 21 IQR 19-26) compared to

controls (median score 2 IQR 0-5), as well as the sleep component of the Brief Pain Inventory interference score (median 8/10 IQR 5-9).

In clinical practice sleep, mood, disability and quality of life should all be assessed in patients with chronic neuropathic pain (Haanä et al., 2011)**.** The findings from this research reinforce the importance of this holistic assessment.

The high scores seen in the Pain Catastrophizing Scale and its components (rumination, magnification and helplessness) may contribute to the severity, chronicity and treatment refractoriness of pain in this cohort. Pain catastrophizing is a robust predictor of pain outcomes, (Sullivan et al., 2001), including chronic neuropathic pain, specifically (Mankovsky et al., 2012; Racine et al., 2016; Lucey et al., 2011; Sullivan et al., 2005). A score greater than 30 is considered clinically relevant on the PCS, and this was met or exceeded in 84% of participants.

It is interesting to consider the results of these measures between this NFCI cohort alongside a cohort with a condition well known to be associated with neuropathic pain, diabetic neuropathy (Themistocleous et al., 2016). These are entirely different conditions with different participant demographics but nevertheless there are some intriguing comparisons (Table 2.7) with similar scores seen for measures of social functioning, and higher scores for pain anxiety and catastrophising.

Another determinant of pain severity and chronicity is any perception of injustice that participants may have held. Perceived injustice has repeatedly been shown to predict adverse

outcomes in chronic pain, and is also correlated to pain catastrophizing. (Sullivan et al., 2012; Sullivan et al., 2011). This was not assessed here but it is highly relevant. That there is perceived injustice is reflected in the fact that 864 claims were made against the Ministry of Defence between 2007 and March 2018 (Kuht et al., 2019). There are several firms of solicitors specialising in bringing claims for NFCI. Clearly such enterprises have a vested interest in reinforcing any sense of injustice and they have a prominent online presence. Other potential perceptions of injustice may derive from perceived institutional bullying or racism.

This study is not the first to consider the role of psychosocial factors in NFCI. Contradictory findings/opinions have been reported throughout the 20th Century, but at a time when attitudes towards social class and psychological disturbance were very different. In 1915 men in whom visible skin changes were not observed, but who complained of sensory symptoms, were described as "jumpy, inattentive and emotional" and the distribution of sensory changes (documented as slipper or stocking distribution) as similar to those found in "hysterical" or "psychical" anaesthesia (Fearnsides, 1915). In 1945 Osborne documented of several of his small cohort that "public morale, in most cases, scarcely existed". Another individual is said to have a "poor personality" (Osborne, 1945). Nevertheless several other authors expressed quite the opposite opinion, perhaps most eloquently so by Redisch: "Medical investigation of these patients (with chronic trench foot) has failed to provide a satisfactory concept of what the condition represents and how the problem may be attacked. However, there is no doubt in the minds of the people who have devoted time to the study of the condition that the men afflicted with chronic trench foot are suffering from a true

Measure	Cases - Median (IQR)	Moderately/Severe Pain in Diabetic Neuropathy (Themistocleous et al., 2016)
SF-36		
SF-36 Physical functioning	60 (25-80)	25 (10-55)
SF-36 Role physical	0 (0-25)	0 (0-50)
SF-36 Bodily pain	22 (12-32)	22 (21.5-41)
SF-36 General health	40 (25-61)	30 (15-45)
SF-36 Vitality	40 (25-50)	40 (25-50)
SF-36 Social functioning	25 (12.5-37.5)	37.5 (25-62.5)
SF-36 Role emotional	0 (0-33.3)	33 (0-100)
SF-36 Mental health	48 (28-64)	60 (48-76)
ISI	21 (19-26)	16 (9-22)
Clinical insomnia	42/51 (82.4%)	54.2%
BPI Interference	7.21 (5.57 – 8.64)	5.5 (4-7.8)
DAPOS		
Depression	15.5 (9-20.25)	8 (5-15)
Anxiety	9 (7-13)	5 (3-8)
Positive outlook	6 (4-8)	10 (7-12)
PCS		
Helplessness	19 (15.25-21)	11 (6-16)
Magnification	10 (6.25-12)	4 (2-8)
Rumination	15 (13-16)	8 (4-13)
Total	43 (35-48)	24 (11.3-33.8)
PASS		
Cognitive	19.5 (14-23)	11.5 (6-19.3)
Escape avoidance	18 (12.75-21)	0 (0-3)
Fear	19 (14-23)	6 (1.8-14.3)
Physiological anxiety	13 (6-18)	3 (0-9)
Total	67.5 (48-78.75)	32 (15.5-55.5)

Table 2.7 Comparison of scores of psychosocial function between NFCI and a different cohort with severe neuropathic pain due to diabetic neuropathy

physical handicap, and that the “psychic element” (usually called upon to cover everything and anything from plain chiselling on the part of the patient to plain ignorance on the part of the physician) is negligible.” (Redisch, 1947). The data in this chapter show beyond doubt that Redisch was correct. Whilst psychological factors are likely to be important, as in any chronic pain condition, there is no doubt of the existence of an underlying “true physical handicap”.

3 Assessment for a genetic contribution to Non freezing cold injury

3.1 Introduction

3.1.1 African and Caribbean individuals are at greater risk of sustaining cold injuries

In April 1917, 1225 cases of "frozen feet" were reported over a two-day period amongst Senegalese troops fighting on the French Western Front, on the side of the allies. During this same period, the number of cases in (presumably) white French troops was negligible (Paton, 2002). Similar observations of a disproportionate number of African/Caribbean casualties have been made repeatedly ever since, during peacetime and in several conflicts, for freezing as well as non-freezing cold injury. Ungley noted in 1942 that "coloured races are said to be more prone to trench foot than northern races" (Ungley, 1942). In the early 21st Century this disparity in predisposition is perhaps most notable (and pertinent to this thesis) in The British Armed Forces, in which personnel from African and Caribbean Commonwealth countries account for excess cases (Burgess & Macfarlane, 2009). It is these individuals that comprise 90% of the cohort in this thesis.

Whilst it does appear clear that African and Caribbean individuals are predisposed to developing cold injury one particularly intriguing, and unanswered, question, is whether or not individuals with darker skin are also more likely to experience late sequelae. There are no published data that address this question. It is interesting to note, however, that in one study pertaining to ethnic predisposition in The British Armed Forces, not only was there a 30-fold increased incidence of cold injury amongst black soldiers, but their injuries were

assessed as more severe than those of white participants (Burgess & Macfarlane, 2009). This may indicate a higher likelihood of progression to late sequelae.

Some studies have attempted to ascertain the reason for the increased propensity to developing *acute* cold injury, focussing on vascular physiological responses to cold (Iampietro et al., 1959a; Maley et al., 2014; Meehan, 1955). These publications have all found that there are differences in the temperatures at which vasoconstriction occurs, as well as skin blood flow measurements, between Caucasian and African individuals, which are predicted to favour the development of cold injuries. As mentioned above, it is also unknown whether there is a specific predisposition to the development of late sequelae of cold injuries in Afro Caribbean individuals, nor whether there are particular differences in phenotype between ethnicities who do progress to this stage.

3.1.2 The genetic contribution to neuropathic pain phenotypes

There are several rare neuropathic pain states with a pure genetic cause, displaying Mendelian inheritance patterns. These include disorders where primarily sodium channel gene mutations are causal: inherited erythromelalgia, paroxysmal extreme pain disorder and congenial insensitivity to pain (as discussed in Chapter 1, and reviewed in (Bennett et al., 2019)). Another rare group of diseases characterised by excessive pain or loss of pain are the hereditary sensory and autonomic neuropathies (HSANs), in which mutations in non-sodium channel genes are causal (Rotthier et al., 2012). In all of these conditions the mutations are rare but the effect size is great.

3.1.3 Disease frequencies and phenotypes are different between ethnicities and have environmental and genetic determinants.

Because there is genetic (as well as environmental) heterogeneity between ethnic groups some diseases with a genetic basis, whether through strict Mendelian inheritance patterns or otherwise, are considerably more common, albeit not exclusive in, certain ethnic groups e.g. Tay Sachs disease in Ashkenazi Jews and Sickle Cell disease in Africans. In these two examples there is a large effect size with a clear phenotype (i.e. as observed in the rare neuropathic pain states mentioned above). It is clear, however, that there is a much more nuanced genetic contribution to many diseases that show variability between ethnicities, where variants act as risk factors rather than causal entities. A variant that is protective in one population may have adverse effects in another. For example, Huang *et al.* found that the Leu72Met ghrelin gene polymorphism was associated with a reduced risk of type 2 diabetes in Caucasians, whilst conferring an increased risk in Asians (Huang et al., 2018). Noting the much higher prevalence of end stage renal disease (ESRD) in African Americans compared to European Americans Kao *et al* investigated the relative frequencies of susceptibility alleles for ESRD and found a highly significant association between several SNPs in nonmuscle myosin heavy chain type II isoform A, MYH9, accounting for a large proportion of the excess risk in African Americans (Kao et al., 2008).

Differences in the observed inter-ethnic frequencies of variants in any given gene are unlikely to be the sole explanation for differences in disease frequencies or phenotypes. For example the association between variant and risk may be different between populations or the importance of gene-environment interactions may vary between ethnicities, such as has been shown for cigarette smoking and lung cancer risk (Haiman et al., 2006). This

consideration is very important in relation to a condition such as NFCI, where the environmental conditions, particularly climate, are very different for individuals from Africa or the Caribbean compared to Europe.

3.1.4 Differences in pain phenotypes are observed between ethnicities

With reference to neuropathic pain, specifically, in a UK study 15,692 diabetes patients of mixed ethnicities, South Asians were found to have significantly higher rates of *painful* diabetic neuropathy compared to Europeans or African Caribbeans (Abbott et al., 2011). In a cross-sectional study of 438 AIDS patients in New York City Breitbart *et al.* observed that pain intensity was significantly higher in non-Caucasian individuals (Breitbart et al., 1996). Similarly, reporting of postoperative pain has been found to be more frequent in African American individuals that Caucasians (Perry et al., 2019) but little work has been conducted to understand any underlying ethnicity-specific genetic component (Somogyi et al., 2017; Somogyi et al., 2016)**.** There is a paucity of data examining the genetic contribution to neuropathic pain in non-Caucasian populations, although there have been some publications addressing this question in Southern African populations with HIV neuropathy. (Hendry et al., 2016; Mbenda et al., 2017; Wadley et al., 2015). Genome-wide association studies (GWAS) are a potentially powerful means to assess for genetic contribution/risk to/for phenotypic traits, particularly where a given trait is common and the genetic effect size is small. In this approach a large cohort of individuals with a given trait is compared, genetically, to a cohort without the trait and a profile can be generated of single nucleotide polymorphisms (SNPs) that are disproportionately found in the 'trait' group. In the field of peripheral neuropathy, for example, this technique has been used to investigate genetic risk of carpal tunnel syndrome using a group of 12,300 patients and comparing them to 390,000

controls (Wiberg et al., 2019). This study identified 16 loci associated with CTS, and strongly implicated genes and biological pathways linked to extracellular matrix architecture. Significant challenges in using GWAS to study neuropathic pain include the relatively small sample sizes available for study, the lack of consensus in definitions of neuropathic pain between studies and the fact that neuropathic pain comprises many different aetiologies all of which will have their own genetic signature (Calvo et al., 2019). In an experimental pain study Hastie *et al.* observed an ethnicity dependent effect of A118G SNP in the mu opioid receptor gene, OPRM 1 (Hastie et al., 2012), such that whilst the presence of the G allele in non-Hispanic white participants was protective, no such effect was observed in Hispanic or African Americans.

3.1.5 Attractive Gene Candidates

The data presented in the previous chapter demonstrated that the pain experienced in the late phase of NFCI is neuropathic in nature and reinforced that cold hypersensitivity is a prominent feature. Therefore genes with known roles in the pathogenesis of neuropathic pain, and especially those involved in noxious cold perception, make particularly attractive candidates for investigation. These include genes, mutations in which are known to cause human disease, as well as those which have not been identified in human disease but, nevertheless, have clear functional impacts when studied *in vitro*.

The molecular basis of innocuous and noxious cold perception has been discussed in greater detail in Chapter 1 of this thesis, including the role of TRPM8, the first cold sensing channel to be identified, in 2002 (McKemy et al., 2002). Whilst the evidence for TRPM8 as a transducer of innocuous cold is unequivocal, its role in noxious cold perception is less certain. Although several pieces of evidence support such a role (Knowlton et al., 2013;

Pogorzala et al., 2013) there are no published reports of mutations in this gene associated with human neuropathic pain states or cold allodynia. However it is interesting to note the correlation with latitude and temperature, of a particular variant upstream of TRPM8, rs10166942, predicted to have arisen due to positive selection (Key et al., 2018), and strongly related to migraine.

Another TRP channel, TRPA1 is also of interest. Heterozygous mutations in this gene are known to cause Familial Episodic Pain Syndrome Type I (Kremeyer et al., 2010). This is a very rare condition, described in only 1 family, but the mutation shows clear segregation with the disease phenotype. A particularly striking feature of the pain experienced in this condition is that one of the potent triggers is cold, also a hallmark of the late stage of NFCI. This protein has also been shown to be crucial for the vascular response to cold (Aubdool et al., 2014). Again, this is particularly interesting in the context of NFCI, where vascular responses to cold may be aberrant (Golden et al., 2013; Ungley et al., 1945). Aubdool *et al* showed that TRPA1 is required for the vasodilator component of the vascular response to cold, a process which has been shown to be attenuated in healthy African individuals compared to white Europeans (Iampietro et al., 1959b), and putatively an aetiological factor in the risk of developing of NFCI.

In contrast to the TRP channel genes, there is a wealth of evidence unequivocally proving the role of sodium channel genes in neuropathic pain states, including congenital insensitivity to pain, paroxysmal extreme pain disorder and inherited erythromelalgia, reviewed extensively by Bennett *et al* (Bennett et al., 2019). Moreover, several members of the family of proteins encoded by these genes, the voltage gated sodium channels, have also been implicated in noxious cold perception. This has been shown, experimentally, for $Na_v1.6$

using axons from mice lacking functional $Na_v1.6$ (Sittl et al., 2012), $Na_v1.8$ (Zimmermann et al., 2007) and $Na_v1.9$ (Lolignier et al., 2015). To date there are no such data for $Na_v1.7$, encoded by *SCN9A*. However, Sereno *et al* have demonstrated a protective effect for a particular polymorphism, rs6746030, in the risk of developing oxaliplatin induced neurotoxicity, a cardinal feature of which is cold allodynia (Sereno et al., 2017). Leipold *et al* have described a three generation family with a striking pain phenotype, including cold aggravated pain, in which there is segregation of a missense change, V1184A in Nav1.9, with phenotype (Leipold et al., 2015).

It seems highly unlikely that any one genetic candidate, or even one family of genes, is solely responsible for the perception of pain at cold temperatures, nor for the phenomenon of cold allodynia.

As detailed in the Methods section of this chapter certain externally imposed constraints meant that it has not been possible to investigate all such genes. Nevertheless it was still possible to investigate potential roles of several attractive candidates (see list in Methods section), including the TRP channels, TRPM8 and TRPA1, as well as sodium and potassium channel genes.

3.2 Materials and methods

3.2.1 Procurement of genetic material

Blood was taken by venepuncture from 72 participants consenting to collection and analysis of genetic material. Samples from the first 40 participants were sent to the Illumina Centre, Cambridge, UK for whole genome sequencing (WGS) as part of a larger project (BRIDGE

Neuropathic Pain (NIHR Pain Consortium)), which enrolled 200 individuals with a variety of neuropathic pain conditions. The BRIDGE consortium has been established as an organisational umbrella for NIHR BioResource funded Next Generation Sequencing (NGS) projects which aim (i) to discover sequence variants underlying hitherto unresolved inherited Rare Diseases or (ii) to evaluate the sensitivity and specificity of exome sequencing or other NGS approaches to identify already known high penetrance rare disease-causing variants.

The reason that only 40 of the 72 samples were sent to The Illumina Centre, Cambridge, was that the NIHR Biorescouces study ended before all samples could be submitted.

After analysis of the results obtained from Whole Genome Sequencing, whole blood from the same individuals was sent to Dr Carl Fratter (Oxford Genetics Laboratories, Oxford University Hospitals NHS Foundation Trust, Churchill Hospital, Oxford), who performed Sanger Sequencing of *SCN9A*.

3.2.2 Handling of samples from 32/72 participants whose blood was not sent for analysis at The Illumina Centre

On the day of venepuncture blood was stored at -80°C until DNA extraction was performed. Samples were thawed at room temperature and then loaded onto the Maxwell® 16 Instrument (Promega AS4500), an automated DNA extraction platform. Automated steps comprised initial cell lysis followed by binding of nucleic acids to paramagnetic particles.

Following automated wash steps the DNA product was eluted in ddH20. This DNA was delivered to Dr Carl Fratter (as above) for Sanger Sequencing of *SCN9A*

3.2.3 Curation of a list of candidate genes

A list of genes pertinent to neuropathic pain was collated and stratified into three tiers according to the strength of evidence for each given gene. It was predetermined that only these genes would be reported by The Consortium. The criteria for stratification were prescribed by the BRIDGE project: Tier 1 genes were those appearing in three high impact publications involved in Neuropathic Pain Disorder (NPD). Tier 2 included those genes appearing in fewer than three high impact publications but nonetheless proven to be involved in neuropathic pain. Tier 3 genes were those without any publications but accepted by the internal community of peers as being implicated in neuropathic pain **(Table 3.1)**

3.2.4 Assignment of putative pathogenicity of variants identified

Single nucleotide variants were only shortlisted if 1) minor allele frequencies were either less than 1 in 1000 for putative novel causal variants, or less than 25 in 1000 for variants already listed as disease causing in The Human Gene Mutation Database (HGMD) (2) they were predicted to have ‘high’ or ‘moderate’ impact according to the Variant Effect Predictor (McLaren et al., 2016) and (3) the variant was present in a relevant gene (Turro et al., 2020)

The potential relevance of variants identified in genes from this list was decided in the setting of multidisciplinary team meetings comprising an experienced clinician with specific knowledge in neuropathic pain (Professor David Bennett, University of Oxford), clinical research fellows who collated phenotypic data (Dr Andreas Themistocleous and Dr Tom Vale, University of Oxford) a scientist with experience in clinical genomics (Natalie van Zuydam, University of Oxford), a clinical bioinformatician (Dr Georgios Baskozos, University of Oxford), electrophysiologists (Dr Iulia Blesneac and Dr Maddalena Comini, University of Oxford) and co-ordinated by a geneticist (Dr Karyn Magy University of Cambridge) (Turro et al., 2020). The likelihood of pathogenicity was ascribed according to guidelines of The American Society of Medical Genetics (Richards et al., 2015)**.** Several factors were considered, including the frequency of the variant in control populations (minor allele frequency of less than one in a thousand), the potential consequences of the variant (e.g. whether or not protein truncating, position in important functional domains, affecting highly conserved amino acids, important amino acid exchange, predicted pathogenic by in silico algorithm) and whether or not the variant had been described, previously, in neuropathic pain conditions. Variants could be determined to be pathogenic, likely pathogenic or of uncertain significance.

Approved HGNC Gene symbol	Tier	Approved Gene name	Disorder (from NPD community)	NPD - applicable disorder from OMIM
FAM134B	Tier 1	family with sequence similarity 134 member B	HSAN 2B;	Hereditary sensory and autonomic neuropathy type IIB
IKBKAP	Tier 1	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein	Riley Day Syndrome/HSAN 3/Familial Dysautonomia	Familial dysautonomia
NGF	Tier 1	nerve growth factor	HSAN 5	Hereditary sensory neuropathy type V
NTRK1	Tier 1	neurotrophic receptor tyrosine kinase 1	HSAN 4/Congenital insensitivity to pain with anhidrosis/CIPA	Congenital insensitivity to pain with anhidrosis (CIPA) also known as Hereditary sensory neuropathy type IV ?
PRDM12	Tier 1	PR domain 12	HSAN 8/Congenital insensitivity to pain	Hereditary sensory and autonomic neuropathy type VIII
RAB7A	Tier 1	RAB7A, member RAS oncogene family	HSAN1/2B	Charcot-Marie-Tooth disease, type 2B also known as Hereditary motor and sensory neuropathy IIB
SCN10A	Tier 1	sodium voltage-gated channel alpha subunit 10	Painful small fibre neuropathy	Familial episodic pain syndrome-2
SCN11A	Tier 1	sodium voltage-gated channel alpha subunit 11	Familial episodic pain; Insensitivity to pain	Familial episodic pain syndrome-3; Hereditary sensory and autonomic neuropathy type VII
SCN9A	Tier 1	sodium voltage-gated channel alpha subunit 9	Different disorders depending on functional s tatus of gene product: Congenital insensitivity to pain, Primary erythromelalgia, Paroxysmal extreme pain disorder	generalized epilepsy with febrile seizures plus, type 7; primary erythermalgia; Familial febrile seizures-3B; autosomal recessive congenital indifference to pain; autosomal recessive hereditary sensory neuropathy type IID; paroxysmal extreme pain disorder; Small fiber neuropathy
SEPT9	Tier 1	septin 9	Hereditary neuralgic amyotrophy	Hereditary neuralgic amyotrophy
SPTLC1	Tier 1	serine palmitoyltransferase long chain base subunit 1	HSAN 1	Hereditary sensory neuropathy type IA
SPTLC2	Tier 1	serine palmitoyltransferase long chain base subunit 2	HSAN 1	Hereditary sensory and autonomic neuropathy type IC
TTR	Tier 1	transthyretin	Familial amyloidosis	Hereditary amyloidosis
WNK1	Tier 1	WNK lysine deficient protein kinase 1	HSAN 2	Hereditary sensory and autonomic neuropathy type IIA;

Approved HGNC Gene symbol	Tier	Approved Gene name	Disorder (from NPD community)	NPD - applicable disorder from OMIM
ATL1	Tier 2	atlastin GTPase 1	HSAN 1; Hereditary Spastic Paraplegia	Hereditary sensory neuropathy type ID; Autosomal dominant spastic paraplegia-3A
ATL3	Tier 2	atlastin GTPase 3	HSAN 1	Hereditary sensory neuropathy type IF
CCT5	Tier 2	chaperonin containing TCP1 subunit 5	HSAN with spastic paraplegia	Autosomal recessive sensory neuropathy with spastic paraplegia
CLTCL1	Tier 2	clathrin heavy chain like 1	HSAN 9/Congenital insensitivity to pain	n/a
KIF1A	Tier 2	kinesin family member 1A	HSAN 2; Hereditary Spastic Paraplegia	Hereditary sensory neuropathy type IIC
MPV17	Tier 2	MPV17, mitochondrial inner membrane protein	Congenital neuropathy leads to absent pain at birth in severe cases. However hepatic failure and encephalopathy overshadow the neuropathy	Mitochondrial DNA depletion syndrome 6 (hepatocerebral type)
NAGLU	Tier 2	N-acetyl-alpha-glucosaminidase	Painful axonal polyneuropathy in heterozygotes; mucopolysacharidosis IIIB when homozygous	Mucopolysaccharidosis type IIIB
TRPA1	Tier 2	transient receptor potential cation channel subfamily A member 1	Familial episodic pain syndrome	Familial episodic pain syndrome-1
KCNA1	Tier 3	potassium voltage-gated channel subfamily A member 1	n/a	Episodic ataxia/myokymia syndrome?
KCNS1	Tier 3	potassium voltage-gated channel modifier subfamily S member 1	No specific Medellian disorder but the rs734784 allele dose dependently enhances risk of nerve pain following nerve injury	Risk enhancer of nerve pain following nerve injury
SCN8A	Tier 3	sodium voltage-gated channel alpha subunit 8	n/a	?
SETDB2	Tier 3	SET domain bifurcated 2	None published but mutation identified in this group: HSN/Insensitivity to pain	n/a
TRPM8	Tier 3	transient receptor potential cation channel subfamily M member 8	n/a	n/a
TRPV1	Tier 3	transient receptor potential cation channel subfamily V member 1	None described	n/a
TRPV2	Tier 3	transient receptor potential cation channel subfamily V member 2	n/a	?
TRPV3	Tier 3	transient receptor potential cation channel subfamily V member 3	n/a	?
TRPV4	Tier 3	transient receptor potential cation channel subfamily V member 4	n/a	?

Table 3.1 List of neuropathic pain genes of interest, ranked into tiers according to level of evidence (as in Methods), with associated disorders NPD Neuropathic Pain Disorder; OMIM Online Mendelian Inheritance in Man

3.2.5 Plasmids and site-directed mutagenesis.

Human $Na_v1.7$ cDNA was cloned into a modified pcDNA3 expression vector containing downstream IRES and dsRED2 sequences (*SCN9A*-IRES-DsRED). Human β1 and β2 subunits were cloned into pIRES2-AcGFP (SCN1B-IRES-SCN2B-IRES-eGFP) (Cox et al., 2006). The mutation R185H was introduced using QuikChange II XL site-directed mutagenesis kit (Agilent).

3.2.6 HEK293T cell culture and transfection.

Human embryonic kidney HEK-293T cells were grown in a Dulbecco's modified Eagle's culture medium (DMEM/F-12, Invitrogen) containing 10% fetal bovine serum and maintained under standard conditions at 37 °C in a humidified atmosphere containing 5% CO2. Cells were transfected using the jetPEI™ transfection reagent (Polyplus-transfection Inc.) with either WT or mutant $Na_v1.7$ channel combined with β1 and β2 subunits (2:1 ratio). Cells were used 36 to 72 hours after transfection.

3.2.7 Electrophysiology.

Patch clamp experiments were performed by Dr Iulia Blesneac, a post-doctoral scientist in Professor David Bennett's Neural Injury Research Group, Nuffield Department of Clinical Neurosciences, University of Oxford. Whole-cell patch clamp recordings were conducted using an Axopatch 200B Amplifier, the Digidata 1550B Low Noise Data Acquisition System and the pClamp10.6 software (Molecular Devices). Data were filtered at 5kHz and digitized at 20kHz. Capacity transients were cancelled and series resistance compensated at 70-90% in all experiments. Voltage clamp experiments were performed on transfected HEK293T cells. The extracellular solutions contained (in mM): 140 NaCl,3 KCl, 1 $CaCl_2$,

1 $MgCl_2$, 10 HEPES, pH 7.3 with NaOH (adjusted to 320 mOsm/L with glucose). Patch pipettes were filled with an internal solution containing (in mM) 140 CsF, 10 NaCl, 1 EGTA, 10 HEPES, pH 7.3 with CsOH (adjusted to 310 mOsm/L with glucose) and had a typical resistance of 2-3MΩ. Leak currents were subtracted using a P/5 protocol, applied after the test pulse. A holding potential of -100mV and an intersweep interval of 10s was used for all the protocols. Current voltage curves (I-V curves) were fitted using a combined Boltzmann and linear ohmic relationship: $I/I_{max}=G_{max}\ (V_m-E_{rev})/(1+\exp^{(V1/2-Vm)/k})$. Normalized conductance-voltage curves (activation curves) were fitted with a Boltzmann equation $G/Gmax=1/(1+\exp^{(V1/2-Vm)/K})$ were G was calculated as follows $G=I/(V_m-E_{rev})$. Similarly, the steady-state fast inactivation curves were fitted with $I/Imax=1/(1+\exp^{-(V1/2-Vm)/k})$. In all the equations $V_{1/2}$ represents the half-activation and respectively half inactivation potential, V_m is the membrane potential, E_{rev} the reversal potential, k the slope factor, G the conductance and I the current at a given V_m; G_{max} and I_{max} are the maximum conductance and current respectively; R_{in} is the fraction of channels that are resistant to slow inactivation. Measurements were done at 10°C, 20°C and 30°C.

3.3 Results

3.3.1 A variant of interest, R185H, in *SCN9A* was identified by preliminary Whole Genome Sequencing

40 participants consented to whole genome sequencing as part of the NIHR-Bridge neuropathic pain project. The only rare variant identified in the candidate gene list in these participants, and considered for further analysis, according to the criteria described in Materials and Methods, was a substitution c.554G>A (rs73969684), in *SCN9A*. This variant substitutes arginine for histidine at position 185 in *SCN9A*. This is the gene encoding the alpha subunit of the sodium channel, $Na_v1.7$. The variant was identified in 6/40 participants, all of whom were heterozygous (an allele frequency of 7.5%).

Following MDT discussion, with reference to the criteria listed in the Materials and Methods section, the variant was considered of uncertain significance in view of the strong environmental contribution to phenotype The variant is reported in several databases, with allele frequencies ranging from 0.2% to 1.4% (Table 3.2).

3.3.1.1 **R185H is predicted to be pathogenic by bioinformatic tools**

The variant results in a missense amino acid substitution in the linker between D1/S2 and D1/S3, within a voltage sensing domain. The residue is conserved across all voltage gated sodium channels. It is predicted to be "probably damaging" and "deleterious" according to Polyphen and "Sorting Intolerant from Tolerant" (SIFT), respectively.

Database	Allele Frequency
Trans-Omics for Precision Medicine (TOPMed)	0.57%
The Genome Aggregation Database (gnomAD)	0.53% 1.4% when looking at an 'African' subgroup with 24182 alleles
NHLBI Exome Sequencing Project (ESP) Exome Variant Server:	0.57%
1000 Genomes Project	0.64%
In 1000 healthy Dutch individuals "of European ancestry" **(Han *et al.*, 2012)**	0.2%
In a cohort of 719 healthy Ghanaians (399 with malaria, 320 without malaria), included in The MalariaGen Network (Band *et al,* 2019)	0.9%

Table 3.2 Reported allele frequencies of the R185H variant in *SCN9A*.

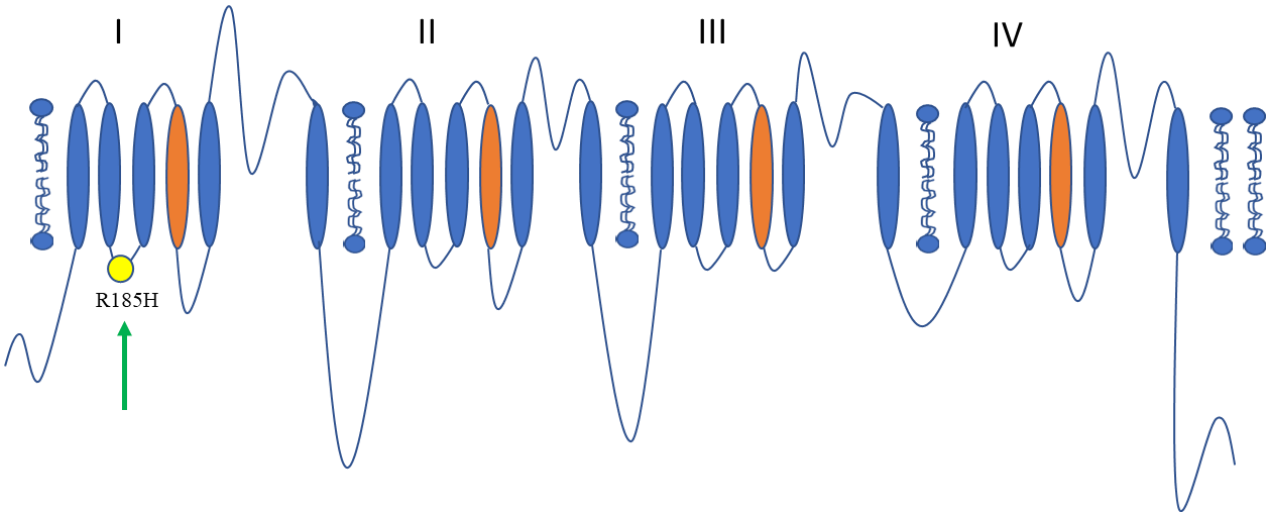


Figure 3.1: Location of the R185H substitution in *SCN9A*

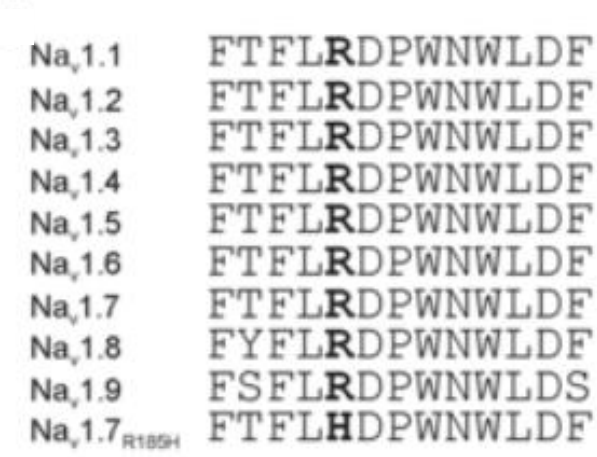


Figure 3.2: The arginine residue at position 185 is conserved across all sodium channels (from Han et al., 2012)

3.3.1.2 The R185H variant was present in a higher than expected proportion of participants

Having identified the R185H variant in 6/40 individuals whose *SCN9A* gene was sequenced as part of The NIHR-BRIDGE project, confirmatory Sanger sequencing of *SCN9A* was performed in these same individuals and in an additional 32 participants. The R185H variant was confirmed in those found to be heterozygous by The NIHR-BRIDGE project in all cases. Of the additional 32 participants, one was found to be heterozygous for R185H. Once these datasets were combined the R185H variant was present in 7/72 participants.

ID	Country of birth	Sex	Age	DN4 score	BPI severity	Sensory sumscore	IENFD
1	Ghana	F	35	8	5.25	18	1.3
2	Cameroon	M	39	6	5.25	10	2.2
3	Ghana	M	27	8	8	16	3.3
4	Ghana	F	40	9	9.5	18	1.6
5	Ghana	M	40	6	3.25	8	3.0
6	Ghana	M	38	7	8.75	10	3.8
7	Ghana	M	31	8	8	8	4.2
		Mean/Median for R195H heterozygotes	31.7	8 (6-8)*	5.25 (3.25-8.75)*	12.6 (8.3-16.8)#	2.8 (1.8 – 3.8)#
		Mean/Median for R185H negative Ghanaians	35.7	8 (7-9)*	7.25 (5-8)*	9.7 (8.2 -11.2)#	4.33 (3.6 – 5.1)#

Table 3.3: Comparison between R185H participants and Ghanaians without R185H Demographic, pain score and neuropathy parameters

***Median (IQR) #Mean (95%CI)**

The heterozygotes were all African, 6 from Ghana and 1 from Cameroon. The overall allele frequency was 4.86%. The allele frequency in African and Caribbean participants was 7/62 (7/124 alleles = 5.65%). The allele frequency in Ghanaian participants was 6/35 (6/70 alleles

= 8.57%). The reported frequency of the variant in healthy African individuals is 1.4% according to gnomAd, and 0.9% in healthy Ghanaians, specifically, using data obtained from The Malaria Genomic Epidemiology Network (799 individuals in total. Personal communication. SNP data extracted by Dr Gavin Bond and Dr Alex Mentzer (Wellcome Trust Centre for Human Genetics, Nuffield Department of Medicine, University of Oxford, UK). Details of the cohort are published (Band *et al*, 2019)

3.3.2 Cellular Electrophysiology

In view of the higher than expected frequency of the R185H variant in *SCN9A* in NFCI participants, cellular electrophysiology experiments were performed in order to investigate the effect of this variant on $Na_v1.7$ function. To investigate potential gene-environment interaction (i.e. the effect of the variant in a cold environment, pertinent to the study of a condition attributed to cold exposure), experiments were performed at 10°C (cooler than usual), as well as at 20°C and 30°C.

3.3.2.1 The R185H variant does not alter current-voltage relationships (IV curves) compared to wild type

The IV curve illustrates the change in current resulting from a given change in voltage. Depolarising 5mV incremental step changes in voltage from -80mV to -20mV resulted in increased current as voltage moves towards activation potential for $Na_v1.7$. As shown in Figure 3.3A, the presence of the R185H variant does not give rise to any change in current voltage relationships (the IV curve) compared to wild type, at 20°C or at the lower

temperature of at 10°C. There was no significant difference in V1/2 between WT or R185H at 20°C or 10°C. Maximum current (and, therefore, channel opening), occurs at -10mV.

3.3.2.2 **Steady state fast inactivation**

The V50 and slope factors are not significantly different between WT and R185H at 20°C. At 10°C there was a significant increase in slope factor in both the WT and R185H, although not a significant difference in the extent of this increase between WT and R185H. In the R185H cells there is a significant difference in half inactivation potential, V50 (a 13mV depolarising shift from -86.9 ± 1.5 at 20°C to -73.1 ± 2.4 at 10°C, $p<0.0001$) that is not seen in WT. These results reflect a lesser degree of inactivation in $Na_v1.7$ in R185H cells at 10°C (i.e. a gain of function characteristic) (Figure 3.3B)

3.3.2.3 **Inactivation kinetics are slowed at 10°C for WT and R185H cells**

Although there was a significant slowing of inactivation kinetics at 10°C for both the WT and the R185H channels, there was not a significant difference in the degree of slowing between WT and R185H (Figure 3.3E).

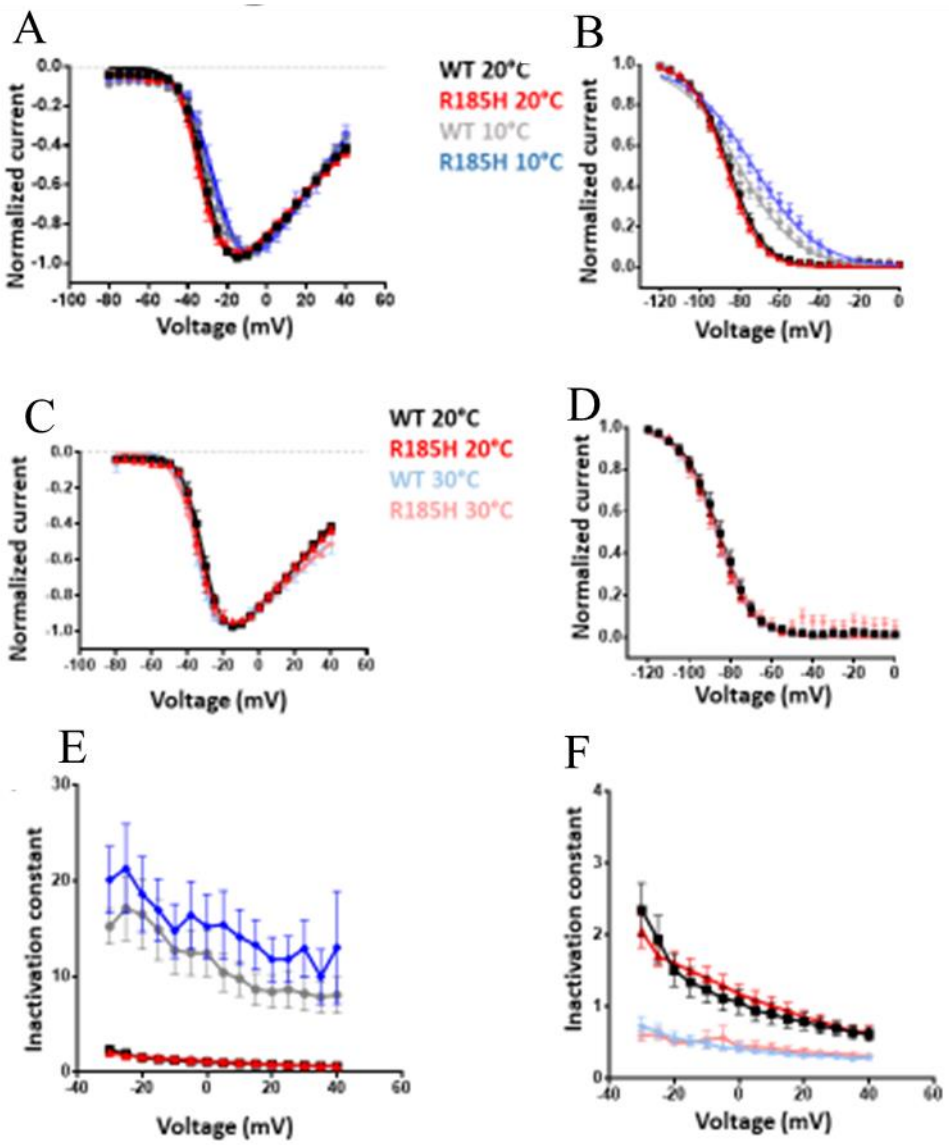


Figure 3.3 R185H displays gain of function characteristics at 10°C but not at 20°C or 30°C

(A) Normalized peak current-voltage relationship curves for the WT at 20°C (black squares, V1/2 = -30.7 ± 1.3, k = 4.9 ± 0.4, n=15), WT at 10°C (grey dots, V1/2 = -26.5 ± 2, k = 6.6 ± 0.6, n=14), R185H at 20°C (red triangles, V1/2 = -31.7 ± 1.7, k = 5 ± 0.5, n=15), R185H at 10°C (blue diamonds, V1/2 = -23.8 ± 1.8, k = 6.7 ± 0.5, n=13). Currents were elicited

from a holding potential of -100 mV to test pulse potentials (50ms) ranging from -80 to 40mV in 5mV increments

(B) Steady-state fast inactivation curves for the WT at 20°C (black squares, V1/2 = -85.8 ± 1.8, k = 7.5 ± 0.6, n=14), WT at 10°C (grey dots, V1/2 = -80 ± 1.8, k = 13.7 ± 0.6, n=13), R185H at 20°C (red triangles, V1/2 = -86.9 ± 1.5, k = 7.6 ± 0.3, n=14), R185H at 10°C (blue diamonds, V1/2 = -73.1 ± 2.4, k = 14.7 ± 0.6, n=13). Currents were elicited with test pulses to -10 mV following 500 ms inactivating prepulses.

(C) Normalized peak current-voltage relationship curves for the WT at 20°C (black squares, V1/2 = -30.7 ± 1.3, k = 4.9 ± 0.4, n=15), WT at 30°C (light blue stars, V1/2 = -34 ± 1, k = 5.2 ± 2.8, n=12), R185H at 20°C (red triangles, V1/2 = -31.7 ± 1.7, k = 5 ± 0.5, n=15), R185H at 30°C (pink asterisks, V1/2 = -33.6 ± 2, k = 4.8 ± 0.5, n=10). Currents were elicited from a holding potential of -100 mV to different test pulse potentials (50ms) ranging from -80 to 40mV in 5mV increments

(D) Steady-state fast inactivation curves for the WT at 20°C (black squares, V1/2 = -85.8 ± 1.8, k = 7.5 ± 0.6, n=14), WT at 30°C (light blue stars, V1/2 = -86.3 ± 2.1, k = 7.5 ± 0.3, n=12), R185H at 20°C (red triangles, V1/2 = -86.9 ± 1.5, k = 7.6 ± 0.3, n=14), R185H at 30°C (pink asterisks, V1/2 = -85.9 ± 2.4, k = 9 ± 1.5, n=9). Currents were elicited with test pulses to -10 mV following 500 ms inactivating prepulses.

(E) Open-state fast-inactivation kinetics for the WT at 20°C (black squares, n=15), WT at 10°C (grey dots, n=14), R185H at 20°C (red triangles, n=15), R185H at 10°C (blue diamonds, n=13).

(F) Open-state fast-inactivation kinetics for WT at 20°C (black squares, n=15), WT at 30°C (light blue stars, n=12), R185H at 20°C (red triangles, n=15), R185H at 30°C (pink asterisks, n=10).

Data are presented as mean ± SEM.

3.4 Discussion

3.4.1 *SCN9A* has a proven role in neuropathic pain

These data support a possible role for a canonical 'neuropathic pain gene', *SCN9A*, encoding the voltage gated sodium channel (VGSC), $Na_v1.7$, in predisposition to the late sequelae of NFCI. This is a highly plausible candidate given its well established role in human pain disorders, including rare conditions demonstrating Mendelian Inheritance: congenital insensitivity to pain (CIP) (Cox et al., 2006), paroxysmal extreme pain disorder (PEPD) (Fertleman et al., 2006), inherited erythromelalgia (IEM) and some small fibre neuropathies that had been previously termed 'idiopathic' (Brouwer et al., 2014).

3.4.2 The R185H variant in *SCN9A* has been reported in neuropathic pain conditions

This variant has been previously reported in a variety of neuropathic pain states, including rare familial cases (see below) as well as cases previously termed 'idiopathic' (Faber, Hoeijmakers, et al., 2012) and in painful diabetic neuropathy (Blesneac et al., 2018)**.** The R185H variant results in a missense amino acid substitution in the linker between D1/S2 and D1/S3, the voltage sensing domain of $Na_v1.7$

Han *et al* described two cases of small fibre neuropathy in patients with R185H (Han et al., 2012). One of these patients was a 54 year old man with typical symptoms of small fibre neuropathy, concordant findings on quantitative sensory testing as well as reduced intraepidermal nerve fibre density. The patient was reported to have family members with symptoms of possible SFN, including painless burn injuries. The other patient was a 24 year old woman, again with typical symptoms, abnormal thermal thresholds and a reduced intraepidermal nerve fibre density. In neither case was genotyping of affected and unaffected

family members performed, and so it is not possible to say whether there was segregation of genotype with phenotype, and no mention of cold exposure as a potential aetiological factor.

In voltage-clamp studies the presence of the R185H variant within DRG neurons was associated with enhanced resurgent currents compared to neurons expressing wild type *SCN9A*, and rendered DRG neurons hyper-excitable in current-clamp experiments. Although no effect on inactivation was observed, these experiments were performed at room temperature (Han et al., 2012) Resurgent currents are persistent, non-inactivating sodium currents that enable spontaneous and high frequency discharges, first described in cerebellar Purkinje cells (Raman & Bean, 1997), and subsequently shown to be generated in DRG cells expressing mutations causal of paroxysmal extreme pain disorder (I1461T in $Na_v1.7$ (Jarecki et al., 2010)) and small fibre neuropathy (G1662S in $Na_v1.8$ (Xiao et al., 2019)).

The variant has also been reported in a three year old child with painful micturition. She would exhibit crying, sweating and writhing, which would all resolve following micturition. Her father (reported to have "some pain in his jaw when yawning") was found to have the same variant. He was unable to recall any episodes of painful voiding, however. These phenotypes are clearly different, and it is questionable whether the father exhibits a truly abnormal phenotype at all (Meglič et al., 2014). Significant improvement in the child's symptoms of painful micturition was reported, coincident with starting carbamazepine. The evidence that the R185H variant was pathogenic in the case is not strong. No details are given regarding the phenotypes of other members of the father's family, and nor was more extensive genotyping performed. Therefore it is not possible to say whether there is co-segregation of phenotype with genotype.

Furthermore, GOF characteristics have been demonstrated for $Na_v1.7$ variants, including R185H, in much commoner neuropathic pain states where the variant is more likely to be acting as a 'risk factor' rather than a causal entity. Faber *et al.* identified variants with GOF properties in 8/28 cases (28.6%) of 'idiopathic' small fibre neuropathy (Faber, Hoeijmakers, et al., 2012) two of whom had the R185H variant. More recently Blesneac *et al*. found rare $Na_v1.7$ variants in 10/111 (9.0 %) individuals with painful diabetic neuropathy. Two of these harboured the R185H variant (Blesneac et al., 2018). Neither of these publications specifically addresses whether or not these individuals had a propensity to cold allodynia.

3.4.3 Expression of the R185H in HEK cells resulted in gain of function characteristics

Gain of function can be brought about either through enhancement of channel activation or through attenuation of inactivation. Sodium channels exist in three states of activation: open, closed and inactivated. In the inactivated state the channel can only transition to a closed state and not back to the open state. This inactivation is mediated by a cluster of hydrophobic amino acids, the IFM (isoleucine-phenylalanine-methionine) motif, which acts to block the open pore of the channel, rendering it impermeable to the inward flow of sodium ions (West et al., 1992).

The electrophysiological experiments performed here revealed certain gain of function characteristics in cells expressing the R185H variant compared to wild type $Na_v1.7$: a low-temperature dependent increase in slope factor, a slowing of inactivation kinetics and a depolarising shift in half inactivation potential. Only this latter parameter was observed to be significantly different from WT for R185H, however.

Gain of function properties at the cellular level (both enhanced activation properties and impaired inactivation properties) have been characterised for a number of neuropathic pain states where sodium channel mutations (not only $Na_v1.7$) are known to be causal.

The specific GOF characteristic demonstrated in the experiments performed here is an impairment of fast inactivation. This mechanism is well documented in paroxysmal extreme pain disorder (Dib-Hajj et al., 2008; Fertleman et al., 2006; Jarecki et al., 2008), a rare condition with a very distinctive neuropathic pain phenotype, in which the causal effect of $Na_v1.7$ mutations is proven. It is feasible, therefore, that this particular electrophysiological alteration could be relevant in NFCI, where pain is also clearly neuropathic in nature, as shown in the previous chapter. Impaired fast inactivation is not the only possible means of rendering nociceptive neurons hyperexcitable. As was shown by Han *et al.* (Han et al., 2012) for the R185H variant, certain mutations causing PEPD have also been shown to lead to the creation of resurgent sodium currents (Jarecki et al., 2010; Theile et al., 2011). Furthermore, whilst enhancement of activation is more commonly seen as the mechanism of gain of function in inherited erythromelalgia, there are pathogenic variants that result in impaired fast inactivation, as for PEPD (e.g. G616R (Choi et al., 2011), A1632T (Eberhardt et al., 2014), and L245V (Emery et al., 2015).

It is much less well described for gain or loss of function characteristics to be temperature dependent, however. Han *et al.* performed similar experiments to those described here (Han et al., 2007) in cells expressing a variant in $Na_v1.7$ known to cause erythromelalgia, L858F. In contrast to the findings in the experiments described in this thesis, Han *et al* found that at lower temperatures (16°C), although there was a depolarising shift in the V50 for steady

state fast inactivation, the magnitude was similar for mutant and WT channels. However, they did find a differential depolarising effect on the V50 of steady state activation. The interpretation of this finding is that at lower temperatures the threshold for activation of L858F channels is higher, causing the gating properties to be more like the WT channels, resulting in a lower probability of firing at lower temperatures relative to warmer temperatures. This would translate into the amelioration of symptoms observed at lower temperatures.

3.4.4 Several sodium channelopathies exhibit temperature dependence on phenotype

Inherited erythromelalgia is well recognised as a sodium channelopathy in which temperature has dramatic effects on symptomatology. The phenomenon is not unique to this condition *or* to $Na_v1.7$, specifically, or even to the nervous system. Several different mutations in SCN4A, encoding $Na_v1.4$, may result in congenital myotonia and periodic paralysis syndromes. One of the striking phenotypic features in paramyotonia congenita is a worsening of myotonia and weakness with cooling. When expressed in HEK cells the P1158S mutation in $Na_v1.4$ has been shown to result in low-temperature dependent hyperpolarising shifts in activation and impairments in slow inactivation (Sugiura et al., 2003; Webb & Cannon, 2008) Similar results have been observed for the Q270K mutation (Carle et al., 2009) and N1366S mutation (Ke et al., 2017). Other conditions in which sodium channel mutations are implicated or proven include febrile seizures, severe myoclonic epilepsy of infancy, long QT syndrome and Brugada syndrome (reviewed in (Egri & Ruben, 2012)), and in each of these conditions temperature dependent variability in phenotype is observed.

Cold allodynia is a symptom described in a variety of painful neuropathies. Some conditions, however, are particularly associated with this phenomenon. These include very rare disorders affecting a single families (e.g. the p.V1184A mutation in $Na_v1.9$ (Leipold et al., 2015)) as well as commoner conditions such as oxaliplatin-induced neurotoxicity and ciguatoxin poisoning. The acute neurotoxicity associated with oxaliplatin chemotherapy is notable for cold-aggravated neuropathic symptoms. The mechanism of this phenomenon is incompletely understood but there is evidence for a role of sodium channels. Sittl *et al.* (Sittl et al., 2012) studied the role of $Na_v1.6$ in oxaliplatin induced neurotoxicity using *in vitro* models. Recordings were made from myelinated and unmyelinated axons, from *Scn8a*$^{med/med}$ mice, which lack functional $Na_v1.6$, as well as in neuronal cell lines transfected with murine $Na_v1.6$. They observed a temperature dependent enhancement of persistent and resurgent sodium currents (as was reported by Han *et al* (Han et al., 2012) in R185H expressing DRG neurons), which was dependent on the presence of functional *Scn8a,* resulting in a slowed time course of fast inactivation. This effect, however, was confined to myelinated fibres and was not seen in unmyelinated C fibres. Although there is little evidence to implicate *SCN9A*, specifically, in the pathogenesis of oxaliplatin-induced neurotoxicity, Sereno *et al.* have observed that a particular missense polymorphism in *SCN9A*, rs6746030, is associated with a protective effect (Sereno et al., 2017). However, caution is required in attributing a role of sodium channels based on this evidence as polymorphisms in many different genes have been implicated (Won et al., 2012). Interestingly another study of South Indian cancer patients found that the same polymorphism was associated with more severe oxaliplatin induced neuropathy (Palugulla et al., 2017), highlighting the potential importance of gene

environment and gene-gene interactions. It may well be the case that whilst sodium channels are important in the pathogenesis of this neuropathy the specific feature of cold-aggravated symptoms is mediated by different mechanisms.

3.4.5 The R185H variant can only be ascribed unknown significance or possible pathogenicity according to published criteria

Whilst a) the over-representation of this variant in the cohort, b) its functional effects *in vitro*¸ and c) its implication in other cases of small fibre neuropathy are of interest, its clinical significance in this cohort is not clear. According to a proposed classification system specifically pertaining to sodium channel gene mutations in pain related disorders, by Waxman *et al* (Waxman et al., 2014), this variant would be ascribed only "possible pathogenicity" (Figure 3.4). This cross-sectional study did not allow for testing of genetic segregation, which would have been very difficult as most of the participants' relatives were not in the UK, and so these cases are best regarded as "sporadic". However predictive algorithms do suggest pathogenicity, which is unsurprising as the variant exists within part of the channel with a known function, the voltage sensing domain.

3.4.6 Variants located near to the 185 locus result in inherited erythromelalgia

There are several other published mutations within domain 1, which have been shown to result in inherited erythromelalgia (IEM). This is of interest given that one of the hallmark clinical features of IEM is the alleviation of pain associated with cooling of the affected extremity (in stark contrast to the pain experienced in NFCI, which is potently evoked by cooling). Three mutations exist in the linker between D1/S4 and D1/S5; I234T, S241T and L245V.

Pathogenic

- Multigenerational segregation with the disease in a family spanning more than three generations, and predictive algorithms unequivocally suggesting pathogenicity
- Multigenerational segregation with the disease in a family spanning more than three generations, and the variant displays gain-of-function changes by voltage-clamp or current-clamp studies, or both
- Segregation with the disease in a nuclear, single-generation family, and predictive algorithms unequivocally suggesting pathogenicity, and the variant displays gain-of-function changes by voltage-clamp and current-clamp studies

Probably pathogenic

- Segregation with the disease in a nuclear, single-generation family and predictive algorithms unequivocally suggesting pathogenicity
- Segregation with the disease in a nuclear, single-generation family and the variant displays gain-of-function changes by voltage-clamp or current-clamp studies

Possibly pathogenic

- Predictive algorithms unequivocally suggest pathogenicity, but segregation cannot be tested or is unclear (e.g. sporadic cases, with possible incomplete penetrance) and no functional studies are available
- Only one family member and the variant displays gain-of-function changes through voltage-clamp or current-clamp studies

Variants with uncertain clinical significance

- Variants for which predictive algorithms suggest pathogenicity, that do not belong to any of the previous categories

Figure 3.4 Classification of pathogenicity of sodium channel variants (Waxman et al., 2014).

3.4.7 There is a rationale to search for variants in additional genes and to further investigate the interaction of the R185H variant with a cold environment

Whilst these results provide a potential mechanism for cold allodynia in a proportion of those inured, the R185H variant was present in only a minority of participants. There may be variants in genes that were not investigated as part of this study. By definition such genes have not been linked to any specific neuropathic pain condition, previously. Differences in

vascular response to cold have been repeatedly demonstrated between ethnicities. Genes encoding the myriad components of this response were not specifically investigated in this work, and clearly variations in those genes could be of fundamental importance. This may be the subject of future research. This discussion has focussed on the potential relevance of R185H to cold allodynia but another consideration is whether this variant, and potentially other gain-of-function variants might predispose to acute injury in the first place. If a variant renders $Na_v1.7$ excessively active at cold temperatures, this could theoretically lead to enhanced sodium load within sensory axons. The $Na_v1.7$ variant, G856D, identified as a cause of small fibre neuropathy, bestows striking gain of function characteristics upon DRG neurons *in vitro* (Hoeijmakers et al., 2012). Additionally, expression of this variant is associated with enhanced persistent sodium currents, causing reverse operation of the sodium/calcium exchanger (NCX2). Instead of transporting calcium out of the cell, it is transported in. When these cells are stressed, metabolically, and depolarised, there is associated neurite fragmentation, which is not seen in wild type cells. This process of degeneration is calcium dependent and, furthermore, can be prevented by the administration of a drug that prevents reversal of the NCX (Estacion et al., 2015). It is possible that R185H could lead to a similar, excitotoxic mechanism of injury. This requires further investigation.

4 Nerve excitability testing in NFCI

4.1 Introduction

In chapter 2, the data demonstrated that there had been injury to small nerve fibres. Despite the deficits seen on clinical examination and quantitative sensory testing suggesting large sensory nerve fibre involvement, nerve conduction studies found no significant abnormality in the nerves tested.

In chapter 3, a sodium channel variant, R185H in SCN9A, was found to be over-represented in the cohort of participants with late sequelae of NFCI. The variant was found exclusively in African individuals. Patch clamp experiments revealed gain of function characteristics when tested at low temperature, providing a potential molecular correlate for the cold allodynia experienced by the participants. These experiments were *in vitro*, where the channel was expressed in non-neuronal cells, outside of the physiological environment in which the sodium channel would normally exert its effects on membrane excitability.

The semi-automated technique of threshold tracking permits the *in vivo* assessment of human axonal excitability. Through the external application of depolarising and hyperpolarising currents of varying magnitudes and durations, different parts of the molecular machinery that modulate axonal excitability can be activated. Based on an understanding of how these processes are normally regulated, inferences can be made regarding membrane potential and ion channel activity. The utility of the technique in the investigation of neuromuscular disorders of many different aetiologies, including genetic,

inflammatory and toxin/drug induced, is well established (Kiernan et al., 2020; Nodera & Kaji, 2006). These include hereditary neuropathies (Charcot Marie Tooth Disease, Fabry's Disease), immune mediated neuropathies (including acute and chronic axonal and demyelinating inflammatory neuropathies), toxic (e.g. chemotherapeutic agent and tetrodotoxin neurotoxicity) and metabolic (e.g. renal disease, diabetes and porphyria) neuropathies. This method of assessment is not in routine clinical use. Historically this was due to a lack of commercially available equipment, but currently because no large-scale trials have been performed to suggest it alters patient management. It does show promise in the prediction of neuropathy in some at risk patient groups (Bae et al., 2011; Park et al., 2009), however, as discussed below. The development of software, which semi-automates the process, providing multiple measures of excitability, has made it considerably easier to undertake (Bostock et al., 1998; Kiernan et al., 2000)**.**

Threshold tracking is well suited to the study of conditions in which perturbances of neuronal ion channel function are known or suspected. As discussed in Chapters 1 and 3, in addition to the rare monogenic neuropathic pain disorders for which numerous ion channel gene mutations have been described, an ever increasing number of publications are reporting on rare variant SNPs in these genes in much commoner disorders such as 'idiopathic' small fibre neuropathy (Faber et al., 2012) and painful diabetic neuropathy (Blesneac et al., 2018). A major caveat to the applicability of threshold tracking techniques to such conditions, however, is that as far as the pain phenotype is concerned, it is the effects of these variants on *small* fibre function which are most directly relevant. There are important differences between large myelinated fibres and small myelinated fibres in terms of their excitability properties (Tigerholm et al., 2020), let alone between large fibres and unmyelinated fibres. Whilst it is possible to perform threshold tracking on small, cutaneous fibres (Hennings et

al., 2017; Hoberg et al., 2019), this is much less well studied and is technically more demanding. The use of threshold tracking techniques in large fibres may still be of benefit in view of the conservation of many ion channels between large and small fibres. Useful insights into the pathophysiology of small fibre dysfunction in various painful neuropathies have been gained from the study of large fibres in patients with these conditions (Bennedsgaard et al., 2020; Farrar et al., 2017; Geevasinga et al., 2012)

The mutations and variants associated with neuropathic pain have frequently been extensively studied *in vitro* but the functional effects *in vivo,* in humans, are much less well characterised. Threshold tracking alone does not provide pure measures of ion channel function, both due to the temporal overlap of the parameters measured and to the influence of other factors (principally membrane potential). When interpreted in the context of existing knowledge of a condition, however, it provides a valuable contribution both to our mechanistic understanding but also as a biomarker. Despite this there are very few publications reporting the use of this technology in the study of neuropathic pain conditions.

It is desirable to understand the mechanisms that may be associated with neuropathic pain *in vivo,* not only to direct further basic scientific research, but also because this can help guide therapeutic options. For example Misawa *et al.* (Misawa et al., 2009) used the technique to find an association of neuropathic pain with increased persistent nodal currents in human subjects with diabetic neuropathy. Subsequently this group administered the sodium blocking analgesic, mexiletine, to diabetic patients with neuropathic pain, observing a suppression of these same currents, correlating with an analgesic effect (Isose et al., 2010). These experiments demonstrate both a possible ionic mechanism for neuropathic pain in this

condition, but also suggest that threshold tracking could potentially be useful as an objective biomarker for analgesic efficacy.

The use of threshold tracking in the study of oxaliplatin neurotoxicity and neuropathy is a useful example to demonstrate the insights and potential clinical benefits the technique can provide. Abnormalities have been shown to predict neuropathy in cancer patients receiving oxaliplatin (Park et al., 2009). Progressive abnormalities developed throughout longitudinal treatment (oxaliplatin is administered at intervals, in multiple doses), before the reduction in sensory amplitudes seen on nerve conduction studies, indicating that this methodology could be useful to risk stratify patients and potentially provide an opportunity to mitigate risk of chronic neuropathy. There was a significant decrease in refractoriness, an effect already shown repeatedly to be due to reduced sodium currents, for example in patients suffering tetrodotoxin (a specific sodium channel blocker) neurotoxicity following ingestion of puffer fish (Matthew C. Kiernan et al., 2005), as well as in patients receiving the sodium channel blocker, mexiletine (Kuwabara et al., 2005). Of relevance to NFCI, this methodology has already been applied to the investigation of cold allodynia, specifically, as a symptom of oxaliplatin neurotoxicity (Bennedsgaard et al., 2020).

The different components of the threshold tracking protocol, what they measure and their significance, will be discussed within the Methods section, which also describes how the automatic software makes these measurements.

4.2 Methods

4.2.1 Set up for experiments

The same room was used for all recordings. In all cases controls and NFCI participants were seated comfortably. The right hand was used for all recordings. The skin was cleaned and exfoliated using fine sandpaper at the sites where electrodes were to be placed (at the wrist, overlying the abductor pollicis brevis muscle, the right index finger, the dorsum of the right hand, and the proximal volar aspect of the forearm). Conventional, non-polarising surface electrodes (3M^{TM} Red Dot^{TM} and Natus® adhesive electrodes) were used for stimulation and recording. The optimum stimulation site was determined as the site at which the lowest threshold was required to elicit maximum response by trial and error using an ADVANCE system (Neurometrix, Massachusetts, USA).

For motor recordings, the recording electrode was placed over the abductor pollicis brevis muscle of the right hand in all cases. The stimulating electrode was placed over the median nerve at the wrist, at the site where maximal CMAP amplitude could be obtained. A reference electrode was placed 10cm proximal to the stimulating electrode (Figure 4.1). For sensory recordings, strip electrodes were placed around the 2nd finger. The stimulating electrode was placed over the median nerve at the wrist, at the site of maximum SNAP amplitude.

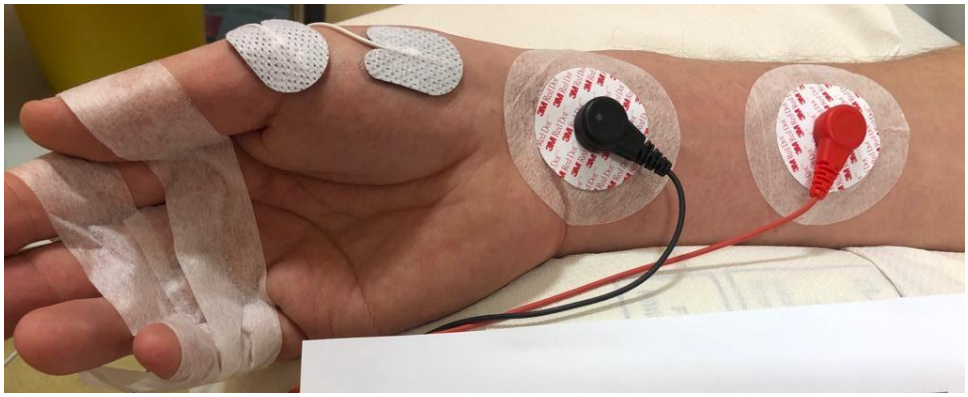


Figure 4.1 Electrode placement for median motor threshold tracking. The positioning of stimulation (black lead), ground (red lead) and recording electrodes is shown.

The median nerve was selected for sensory and motor studies as this is a site where reliable sensory and motor recordings are obtained. This was the nerve used in the development of The TROND protocol (Kiernan et al., 2000). All NFCI participants studied by threshold tracking experienced pain in the hands as well as the feet.

Because various measures in the TROND protocol are sensitive to temperature, skin temperature was measured at the site of stimulation using an infrared thermometer, and kept above 32°C, where necessary, using an infrared heating lamp.

Stimulus current was applied using an isolated linear bipolar constant current stimulator (DS5, Digitimer Ltd). To reduce 50Hz electrical interference, a HumBug (Quest Scientific Instruments, North Vancouver, Canada) noise elimination device was connected between the preamplifier and the data acquisition device (Figure 4.2)

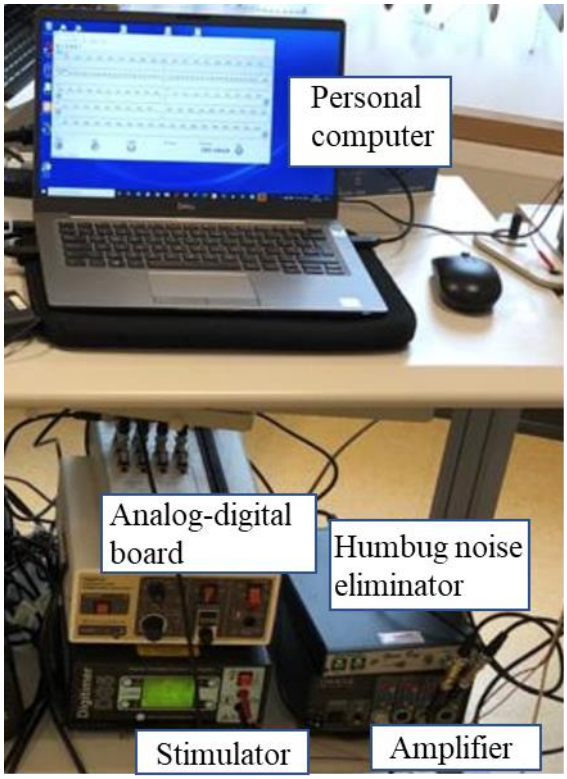


Figure 4.2 Equipment setup for threshold tracking – components labelled.

4.2.2 Delivery of The TROND Protocol

Stimulus intensity was increased manually in 2% increments until a supramaximal response was obtained. The Qtrac software (Institute of Neurology, University College London, London) was then activated to lower the current stimulus until a 40% stimulus response was elicited, which was defined as threshold for the remainder of the sensory and motor experiments, (Figure 4.3). The protocol comprises the following indices: 1) strength duration properties, including rheobase and strength duration time constant (2) current–threshold relationship consisting of the threshold changes after 200 ms of polarizing current, varied between -50% and 100% of control threshold current (3) threshold electrotonus (TE) with 100ms polarizing currents set to ±40% and +20% of control threshold current, and (4) recovery cycle of percentage changes in threshold following a supramaximal stimulus.

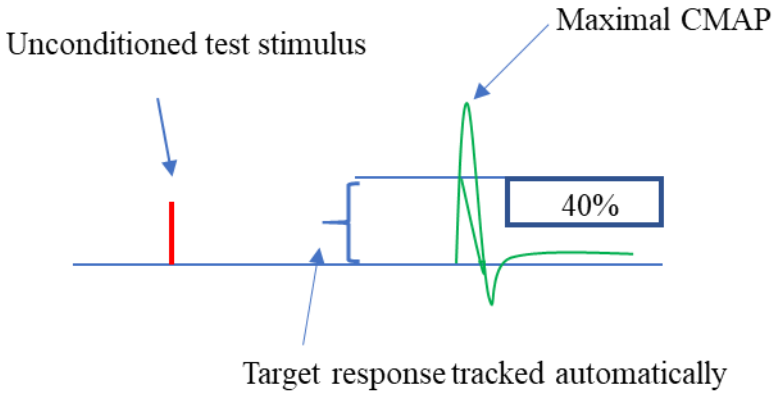


Figure 4.3 Establishing threshold to track. Target response is 40% maximum CMAP (for motor recording) or compound action potential (for sensory recording)

4.2.2.1 **Strength duration properties**

Significance and principal of measurement

These are nodal properties, determined by the activity of persistent sodium currents at the Node of Ranvier (Bostock & Rothwell, 1997)**.** This index of excitability measures the relationship between the duration of a given stimulus, and the current required to obtain threshold response at that duration. According to Weiss's Law there is a linear relationship between stimulus duration and stimulus charge, e.g. if the duration is lengthened the current can be reduced to reach the same thresholds. The reason this is important is that the sodium channels that determine time constant (τ_{SD}) also influence spontaneous and repetitive activity (Kiernan & Lin, 2012)

Stimuli of two durations, 0.2ms and 1.0ms, are applied and the resulting threshold charge stimuli required to elicit threshold, plotted. A line is drawn between the points plotted for the respective stimulus durations. The slope of this line is the rheobase, the threshold current required for a stimulus of infinitely long duration. The point at which the line intercepts the negative X axis is the strength duration time constant (τ_{SD}). The voltage dependence of these measures is due to nodal persistent current (Bostock, 1983; Mogyoros et al., 1996) (Figure 4.4.1).

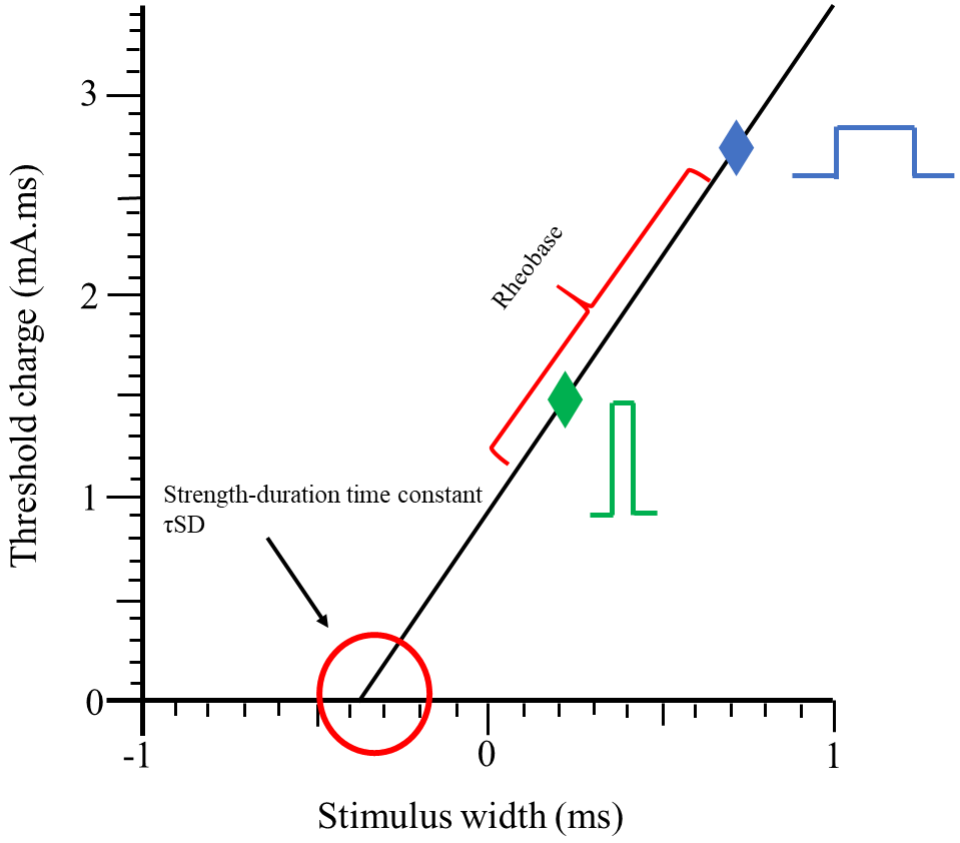


Figure 4.4.1 Current duration vs strength. Stimuli of two durations are delivered. The relationship between strength and duration is linear. The slope of the line is the rheobase. Where the line drawn between the two points intersects the X axis is the strength duration time constant.

Stimulation pattern delivered by Qtrac

Stimulus–response curves were recorded separately for test stimuli of durations 0.2 ms and 1.0ms (Figure 4.4.1). The stimuli were increased in 6% steps, with two responses averaged for each step, until three averages were considered maximal.

4.2.2.2 Current threshold relationship

Significance and principal of measurement

This relationship reflects nodal and internodal rectifying properties. Rectification currents act to fine tune membrane excitability to prevent excessive hyperpolarisation (through inward rectification) or depolarisation (through outward rectification). Prolonged hyperpolarising and depolarising conditioning currents over a range from -100% to +50% potentials, are applied and the effect on test stimulus threshold plotted. Steepening of the curve in the hyperpolarising or depolarising direction reflects increasing rectification i.e. the smaller the change in threshold for an injected current, the greater the rectification. In the depolarising direction this reflects outward rectification, an accommodative response to depolarisation associated with activation of fast and slow potassium channels. The less prominent steepening observed in the hyperpolarising direction is due to inward rectification, mediated by the hyperpolarization-activated conductance through HCN (hyperpolarisation activated cation) channels (Figure 4.4.2)

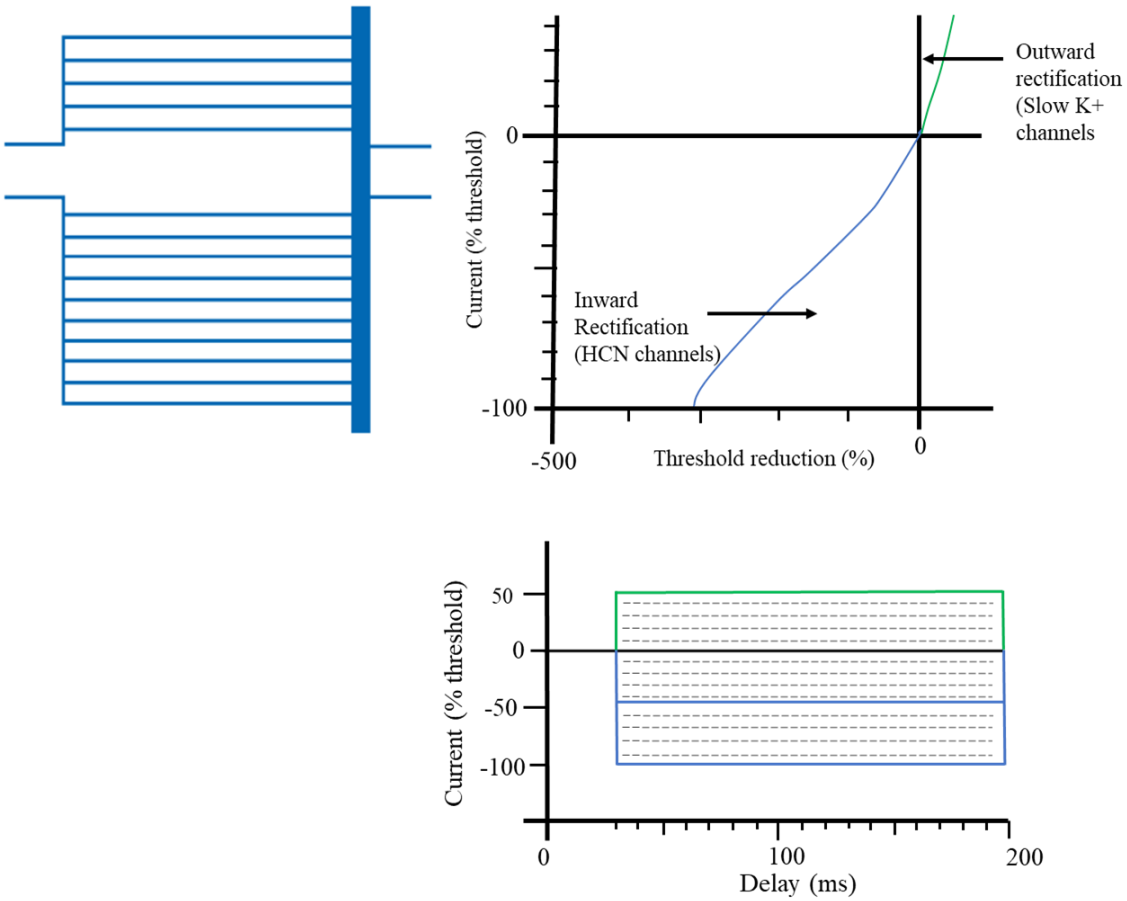


Figure 4.4.2 Current threshold relationship: Top right trace: the effects of prolonged hyperpolarising (negative threshold reduction on the X axis) and depolarising (positive threshold reduction on the X axis) are plotted against the required test stimulus to achieve threshold. Top left and bottom traces: Prolonged conditioning stimuli of varying intensities are applied. Figure adapted from Kiernan & Lin, 2012 and Kiernan et al., 2020.

Stimulation pattern delivered by Qtrac

Sixteen different conditioning stimuli, lasting 200 ms, were applied in increments of 10% from +50 to -100 percent of threshold current. Stimuli with conditioning currents were alternated with test stimuli lasting 1.0ms, alone, and each stimulus combination repeated until three valid threshold estimated were obtained (Figure 4.4.2)

4.2.2.3 **Threshold electrotonus**

Significance and principal of measurement

This part of the protocol examines internodal conductance in relatively depolarised and hyperpolarised membrane states. Depending on whether a subthreshold depolarising or hyperpolarising conditioning stimulus is given, there will be activation of different populations of internodal ion channels. As with the current threshold relationship, threshold electrotonus is a useful measure of the accommodative properties of the axonal membrane. Subthreshold (i.e. too weak to generate action potentials), rather than suprathreshold, conditioning stimuli are applied because it is the axonal events *during* an impulse rather than *after* an impulse, which are of interest.

Electrotonus refers to alteration of potential difference across the internodal membrane, brought about through prolonged subthreshold current application. Different strengths of subthreshold current can be applied, both hyperpolarising and depolarising. Threshold electrotonus refers to changes in threshold associated with electrotonus and reflects underlying changes in membrane potential.

In the case of applied subthreshold *depolarising* current, initially there is rapid membrane depolarisation and threshold falls, quickly. Two periods of accommodation follow due to activation of fast and slow potassium currents, respectively. These currents are dependent upon a) the function of 'their' respective ion channels and b) the equilibrium potential for potassium (itself determined by extracellular and intracellular potassium concentration, and temperature, as predicted by the Nernst Equation). Fast potassium channel activation slows

the rate of depolarisation, reflected in the S1 accommodation phase. Slow potassium channels are responsible for the decline in electrotonus back towards baseline, the S2 phase. When the subthreshold conditioning current ends, there is rapid drop in depolarising electrotonus, reflecting repolarisation at the node. These phases are depicted in Figure 4.4.3.

With subthreshold *hyperpolarising* current there is a rapid increase in threshold (a negative threshold reduction), which is then accommodated by activation of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. Hyperpolarisation also deactivates internodal potassium channels and so these do not act to limit the extent of hyperpolarisation.

The data are conventionally quantified by recording measurements of threshold at different intervals following the depolarising or hyperpolarising stimuli, which are expressed as percentage of threshold. Notation of results reflects the polarity of the stimulus and the time point following stimulation that the recording is measured. For example, the percentage of stimulus required to achieve threshold between 10-20ms after the depolarising stimulus is denoted TEd 10-20. The percentage of stimulus required to achieve threshold 10-20ms after hyperpolarising stimulus is TEh 10-20

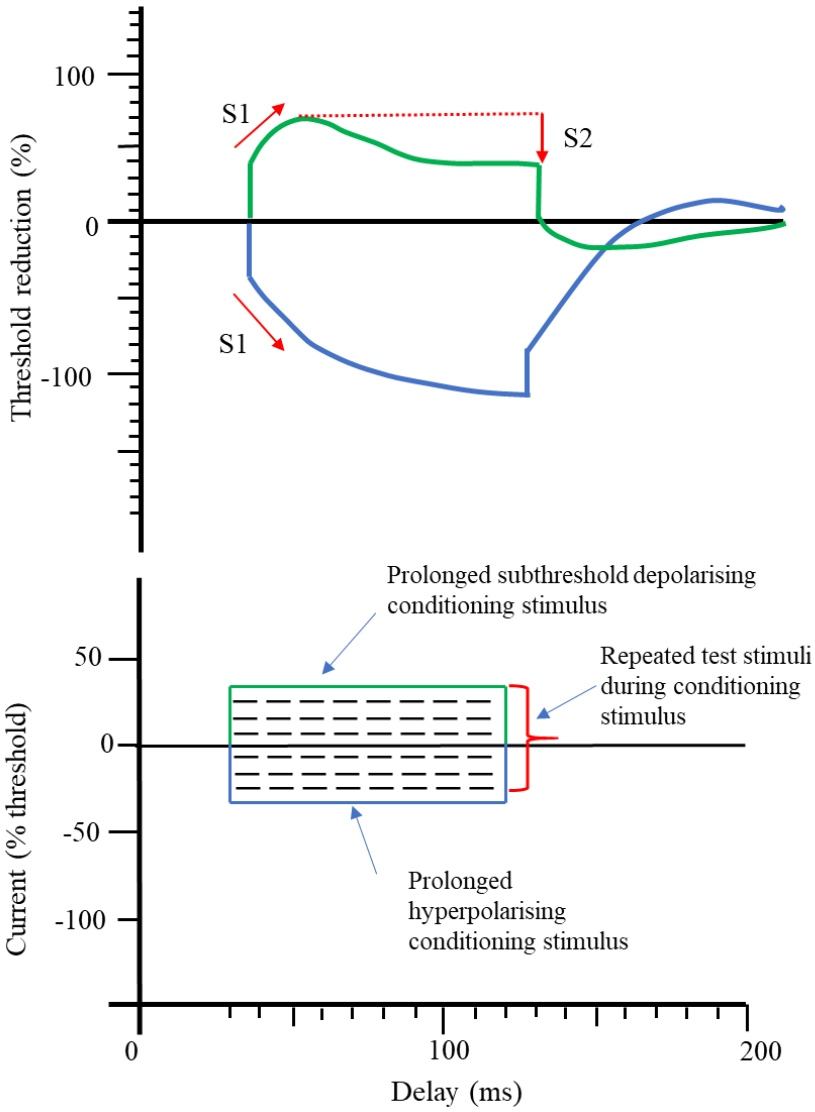


Figure 4.4.3 Threshold electrotonus plot and stimulation patterns. Top trace: Threshold electrotonus plot. Changes in threshold plotted as % reductions, with responses to depolarising stimuli upwards, and to hyperpolarising stimuli, downwards. Bottom trace: Depiction of simultaneous prolonged subthreshold depolarising (upwards) and hyperpolarising (downwards) stimuli.

Stimulation pattern delivered by Qtrac

Prolonged (100 ms) subthreshold depolarising (+20% and +40% of threshold current) and hyperpolarising (-40% of threshold current) currents were used to alter the potential difference across the internodal axonal membrane. Test stimuli of 1-ms duration were used to produce the target CMAP or sensory compound action potential (40% of maximal), and threshold tracking was used to record the changes in threshold. The three stimulus combinations were tested in turn: test stimulus alone (to measure the control threshold current); test stimulus + depolarizing conditioning current; and test stimulus + hyperpolarizing conditioning current. Threshold was tested at 26 timepoints (maximum separation 10 ms) before, during, and after the 100-ms conditioning currents. Each stimulus combination was repeated until three valid threshold estimates were recorded, as judged by the response being within 15% of the target response, or alternate responses being either side of the target (Figure 4.4.3).

4.2.2.4 **Recovery cycle**

Significance and principal of measurement

This index is concerned with the period *after* an impulse has happened, during which the membrane goes through a series of excitability changes. It provides information regarding ion currents, particularly sodium and slow potassium currents (and, inferred from this, ion channel function and equilibrium potentials) and membrane polarity.

The recovery cycle consists of different stages of altered excitability after an action potential. Axons are initially absolutely refractory because all sodium channels are inactivated. No current can flow in no matter how high the stimulus. There follows a period of relative refractoriness (early subnormal), such that they have higher activation thresholds than baseline, because sodium channels have begun to recover. About 3ms later they become hyperexcitable relative to baseline (they are supernormal/superexcitable), during the depolarising afterpotential. This is due to the long-lasting, passive, depolarisation of the internodal axon following the action potential. Then there is a further period of subnormality, the later subnormal period, where threshold is raised once more due to afterhyperpolarisation of the internodal axon when there is potassium ion egress (potassium channels are slower to inactivate than sodium channels). This period is not necessarily a pure surrogate of slow potassium current, however, because it is also influenced by the extent of the preceding superexcitable period

One suprathreshold conditioning stimulus is applied to generate an action potential and thence to initiate the sequence of changes that occurs afterwards. Test stimuli are then delivered at various intervals following the conditioning stimulus (Figure 4.4.4).

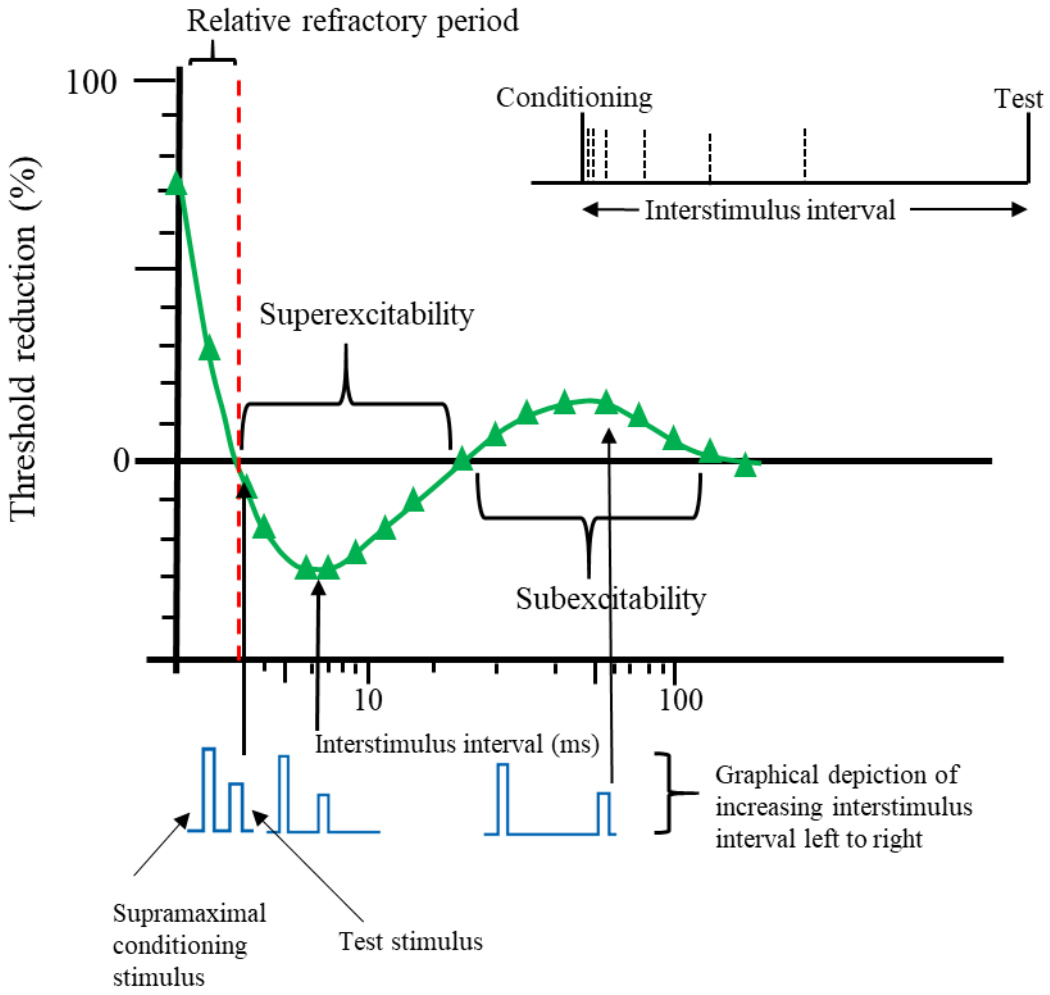


Figure 4.4.4 The recovery cycle. Main plot, the effect upon test stimulus at increasing intervals following a suprathreshold conditioning stimulus. Inset and below main plot: Graphical depictions of interstimulus interval pattern (Figure adapted from Kiernan & Lin, 2012 and Kiernan et al., 2020).

Stimulation pattern delivered by Qtrac

These changes were recorded at 18 conditioning–test intervals, decreasing from 200 to 2 ms in approximately geometric progression. Three stimulus combinations were tested in turn: (1) unconditioned test stimulus (of 1-ms duration) tracking the control threshold; (2) supramaximal conditioning stimulus (1-ms duration) alone; and (3) conditioning + test stimuli. The response to (2) was subtracted on-line from the response to (3) before the test

CMAP/CAP was measured, so that the conditioning maximal CMAP/CAP did not contaminate the measured response when the conditioning test interval was short. Each stimulus combination was repeated until four valid threshold estimates were obtained.

4.2.3 Statistical analysis

In light of the multiple comparisons, a P value < 0.01 was considered significant, as previously used, and recommended in personal communication with Professor Matthew Kiernan, University of Sydney (Kiernan et al., 2001; Park et al., 2014)**.** The five different stimulation protocols were considered independent variables and *not* each individual parameter within each protocol

Unpaired t tests were used for comparison of normally distributed data. Mann Whitney tests were used for comparison of non-parametric data.

4.3 Results

16 NFCI participants underwent sensory recordings (Mean age 31.2±4, 3 white, 1 Caribbean, 12 African, all male). There were 14 control sensory recordings for comparison (Mean age 30.7±7, all white, 9 male)

22 NFCI participants underwent motor recordings (Mean age 30.8±3.9, 4 white, 2 Caribbean, 16 African, all male). There were 9 control motor recordings for comparison (Mean age 30.3±8.3, all white, 6 male)

Discrepancies between the number of NFCI participant recordings and control recordings are due to technical difficulties arising during recording sessions: these included unfavourable signal:noise ratios and excessive skin resistance despite use of the HumBug device to eliminate 50Hz noise and thorough skin preparation, respectively.

4.3.1 Stimulus response and strength duration properties

Sensory: No significant differences were observed for stimulus required for 50% maximal response, SDTC or rheobase (Table 4.1; Figure 4.5.1)

Motor: No significant differences were observed for stimulus required for 50% CMAP amplitude, SDTC or rheobase (Table 4.2; Figure 4.5.2)

4.3.2 Current threshold relationship

Sensory: No significant differences were observed (Table 4.1; Figure 4.5.1)

Motor: No significant differences were observed at any time point (Table 4.2; Figure 4.5.2

4.3.3 Threshold Electrotonus

Sensory: No significant differences were observed at any timepoint during or after conditioning stimuli **(**Table 4.1; Figure 4.5.1).

Motor: There was a small significant reduction in S2 accommodation in NFCI participants compared to controls (p=0.01) (Table 4.2; Figure 4.5.2 and Figure 4.5.2.1).

4.3.4 Recovery Cycle

Sensory: No significant differences were observed in refractory period or superexcitability (Table 4.1; Figure 4.5.1).

Motor: Late subexcitability was significantly reduced in the NFCI group compared to controls 11.37±3.48 *vs* 15.26±1.29, *p* 0.002 (Table 4.2; Figure 4.5.2 and Figure 4.5.2.2).

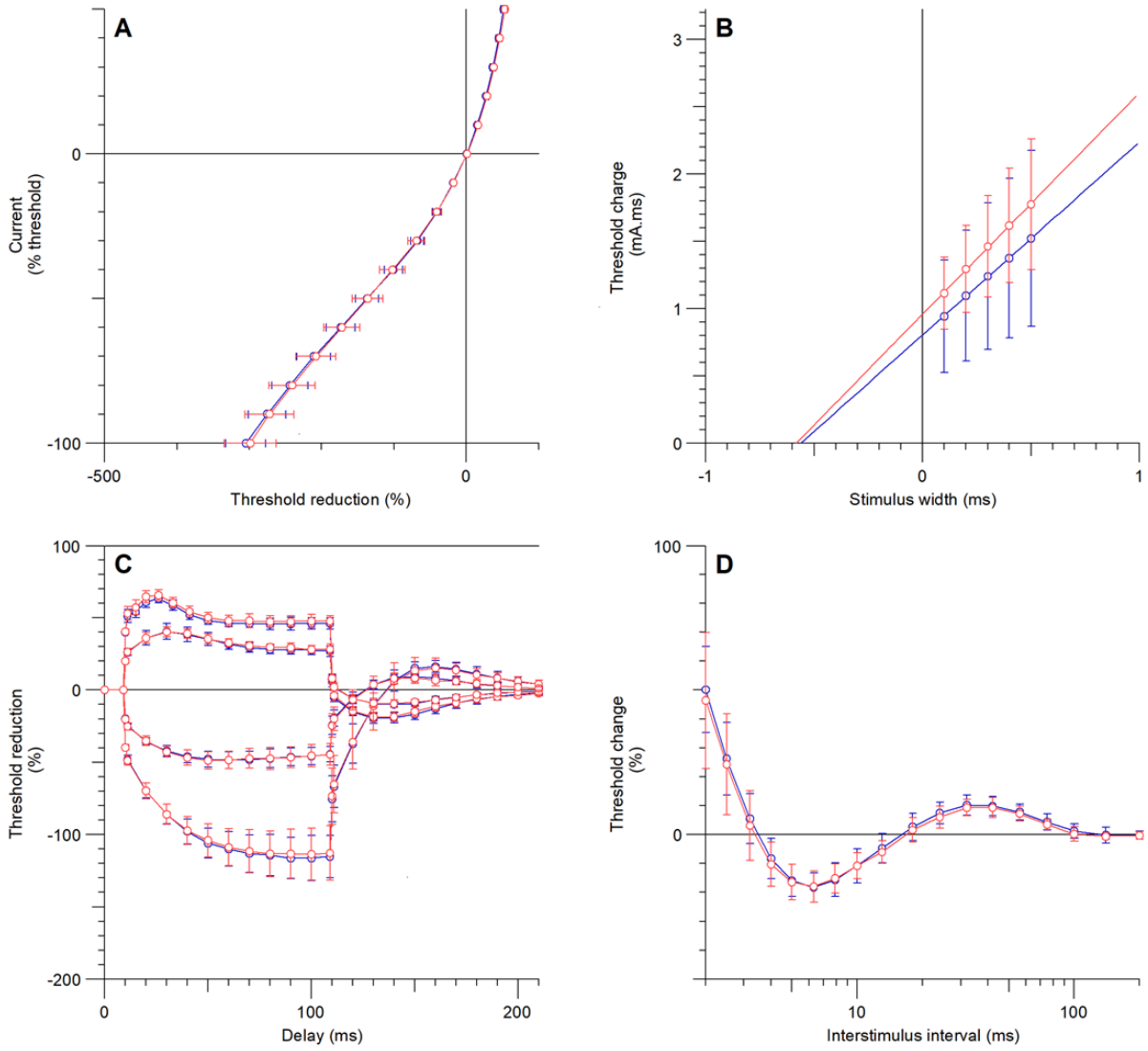


Figure 4.5.1 Plots for threshold tracking of median <u>sensory</u> nerve (A) current threshold relationship, (B) Strength duration properties, (C) threshold electrotonus and (D) recovery cycle. Blue circles represent mean control values. Blue vertical lines represent +/- standard deviation. Red circles represent mean NFCI group values. Red vertical lines represent +/- standard deviation.

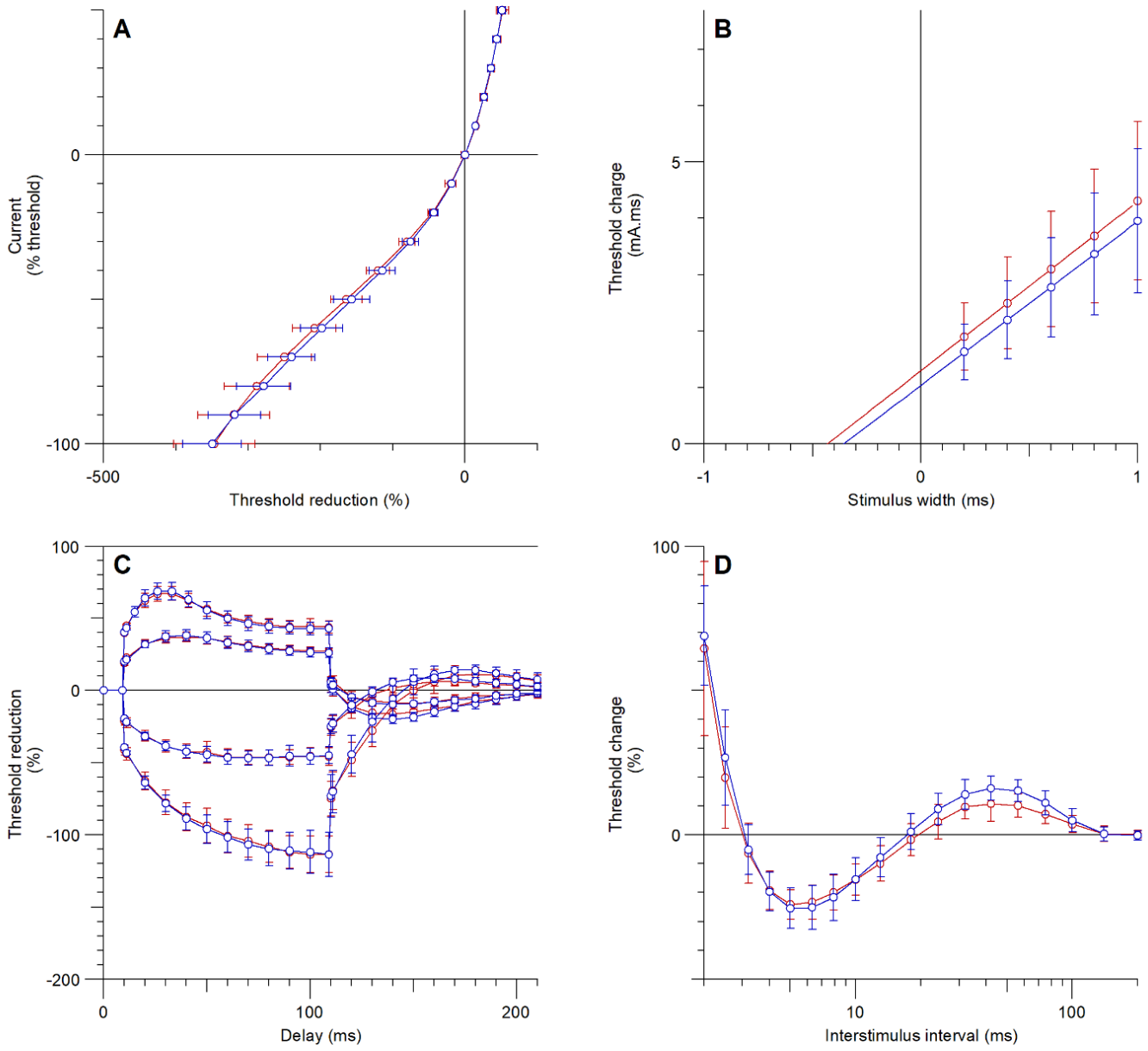


Figure 4.5.2 Plots for threshold tracking of median <u>motor</u> nerve (A) current threshold relationship, (B) Strength duration properties, (C) threshold electrotonus and (D) recovery cycle. Blue circles represent mean control values. Blue vertical lines represent +/- standard deviation. Red circles represent mean NFCI group values. Red vertical lines represent +/- standard deviation.

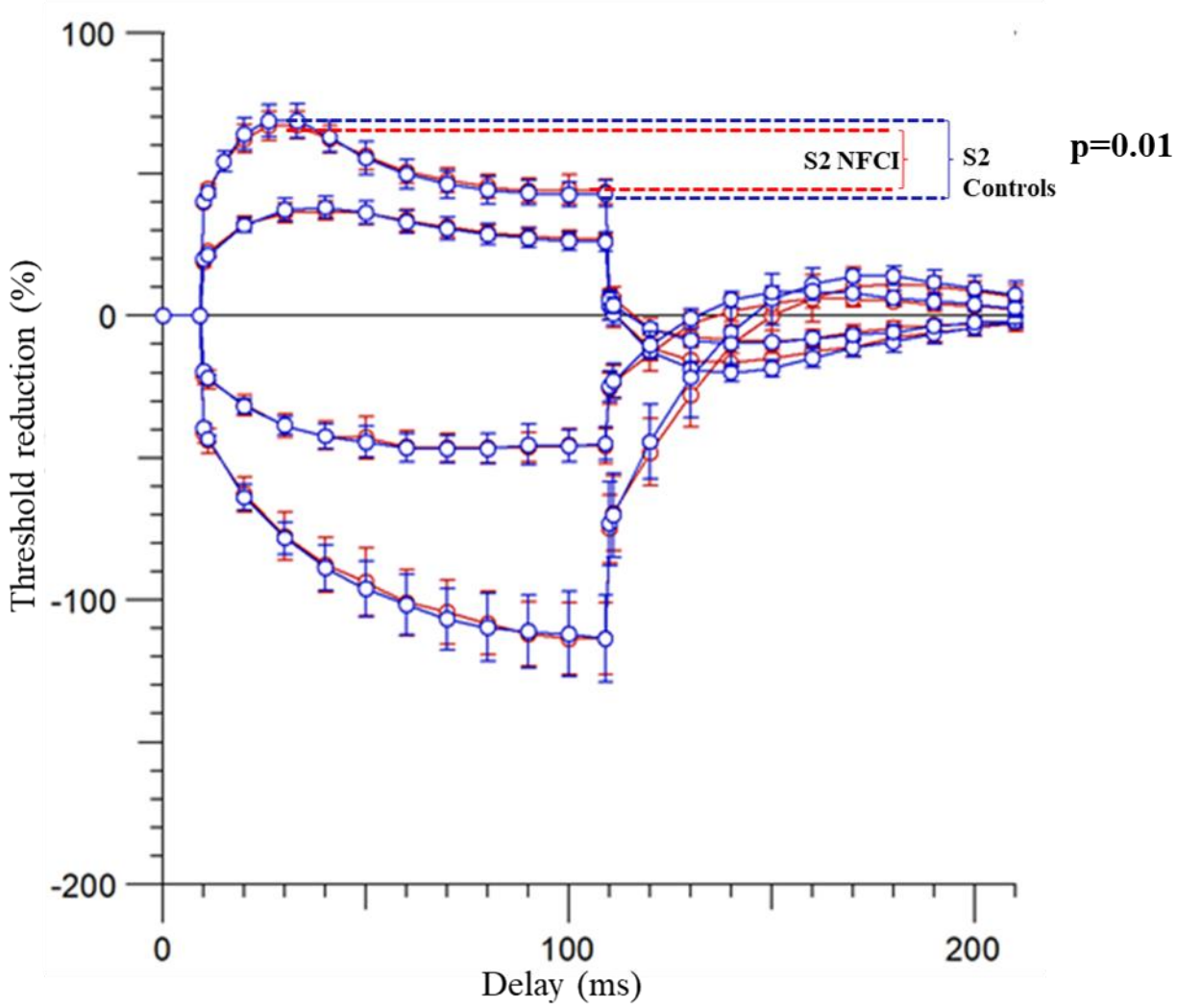


Figure 4.5.2.1: Enlarged plot of threshold electrotonus for median motor nerve. Blue circles represent mean control values. Blue vertical lines represent +/- standard deviation. Red circles represent mean NFCI group values. Red vertical lines represent +/- standard deviation.

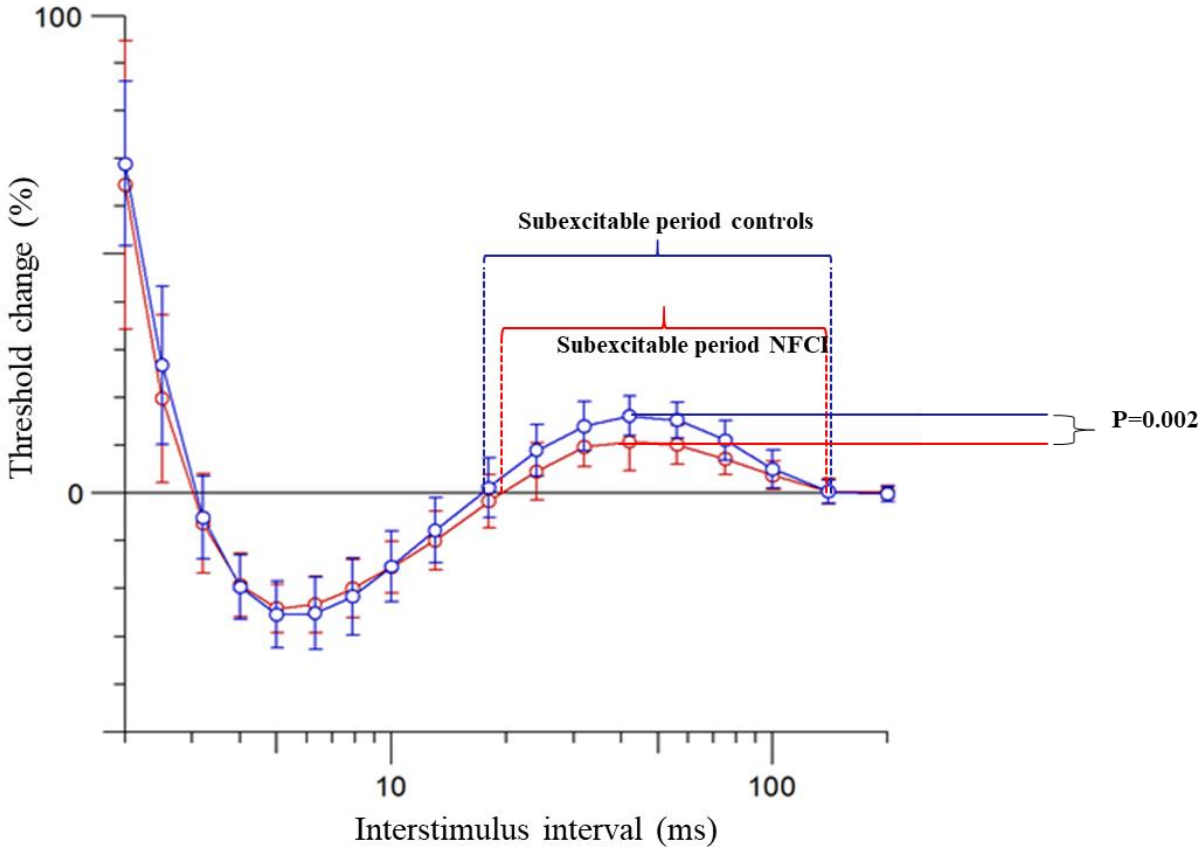


Figure 4.5.2.2: Enlarged plot of recovery cycle for median motor nerve. Blue circles represent mean control values. Blue vertical lines represent +/- standard deviation. Red circles represent mean NFCI group values. Red vertical lines represent +/- standard deviation.

Sensory	Healthy Controls (n = 14) (SD)	NFCI Participants (n = 16) (SD)	Controls vs NFCI (p value)
Stimulus response and strength duration properties			
Stimulus required for 50% maximum response (mA)	3.54 (1.29)	3.71 (0.89)	.66
SDTC (ms)	0.55 (0.01)	0.61 (0.10)	.10
Rheobase (mA)	1.59 (0.65)	1.65 (0.57)	.81
Threshold Electrotonus			
TEd 10-20ms (%)	62.37 (2.80)	64.55 (3.40)	.07
TEd Peak (%)	60.65 (2.63)	62.71 (3.42)	.08
TEd 40-60ms (%)	46.69 (3.79)	48.39 (3.85)	.23
TEd 90-100ms (%)	45.84 (5.00)	47.73 (4.05)	.26
S2 Accommodation (%)	14.82 (3.76)	14.98 (3.01)	.89
TEd^{20} Peak (%)	39.79 (4.74)	39.24 (3.07)	.71
TEd^{40} Accommodation (%)	15.45 (3.71)	15.86 (2.92)	.74
TEh 10-20ms (%)	-78.50 (6.09)	-77.87 (6.09)	.78
TEh 20-40ms (%)	-97.98 (8.33)	-96.11 (9.17)	.57
TEh 90-100ms (%)	-118.40 (15.63)	-113.20 (18.38)	.42
Accommodation Half Time (ms)	30.79 (3.91)	29.83 (3.52)	.48
Recovery Cycle			
Relative refractory period (ms)	3.47 (0.40)	3.32 (0.51)	.40
Superexcitability (%)	-18.28 (4.72)	-16.54 (5.40)	.37
Subexcitability (%)	10.25 (1.43)	9.05 (2.77)	.13
Refractoriness at 2.5ms (%)	25.63 (12.92)	24.25 (17.45)	.81

Table 4.1 Mean excitability values median sensory nerve for healthy controls and the NFCI group. Data shown for Stimulus response and strength duration properties, threshold electrotonus and recovery cycle parameters. TEd = depolarising electrotonus. TEh = hyperpolarising electrotonus. Values compared using unpaired t test.

SDTC = Strength duration time constant

Motor	Healthy Controls (n=9) (SD)	NFCI Participants (n=22) (SD)	Controls vs NFCI (p value)
Stimulus response and strength duration properties			
Stimulus required for 50% max CMAP (mA)	4.15 (1.3)	4.41 (1.4)	.60
SDTC (ms)	0.36 (0.05)	0.44 (0.10)	.09
Rheobase (mA)	2.91 (0.99)	3.00 (1.03)	.82
Threshold electrotonus			
TEd 10-20ms (%)	67.16 (5.61)	65.38 (4.84)	.38
TEd Peak (%)	67.16 (5.56)	65.79 (4.46)	.48
TEd 40-60ms (%)	50.31 (5.18)	51.37 (4.30)	.56
TEd 90-100ms (%)	42.81 (4.54)	44.12 (4.33)	.89
S2 Accommodation (%)	24.35 (1.82)	21.67 (2.78)	**.01**
TEd^{20} Peak (%)	37.55 (3.84)	37.11 (3.53)	.76
TEd^{40} Accommodation (%)	24.31 (1.81)	21.70 (2.83)	.05
TEh 10-20ms (%)	-71.10 (4.67)	-70.25 (7.16)	.74
TEh 20-40ms (%)	-87.87 (7.73)	-86.56 (9.69)	.73
TEh 90-100ms (%)	-112.80 (14.9)	-113.5 (12.38)	.89
Accommodation Half Time (ms)	41.39 (5.79)	42.38 (5.33)	.65
Recovery cycle			
Relative refractory period (ms)	3.06 (0.26)	2.98 (0.39)	.56
Superexcitability (%)	-24.66 ((7.19)	-23.40 (5.32)	.59
Subexcitability (%)	15.26 (3.88)	11.37 (3.48)	**.002**
Refractoriness at 2.5m (%)	26.72 (16.51)	19.72 (17.58)	.31

Table 4.2 Mean excitability values median motor nerve for healthy controls and the NFCI group. Data shown for Stimulus response and strength duration properties, threshold electrotonus and recovery cycle parameters. TEd = depolarising electrotonus. TEh = hyperpolarising electrotonus. Values compared using unpaired t test.

SDTC = Strength duration time constant

4.4 Discussion

4.4.1 Main findings

The data from these threshold tracking experiments demonstrate a significant reduction in late subexcitability of the recovery cycle in the NFCI group compared to healthy controls. There is also a small significant reduction in the S2 accommodative phase of depolarising electrotonus. These indices reflect changes in slow potassium current (Kiernan et al., 2020)**,** suggesting a potential mechanism for axonal hyperexcitability. However, caution is required because no individual ion channel is uniquely responsible for a single process. Changes in one phase may have downstream effects on a later phase. For example changes in the superexcitable period will alter threshold during the ensuing subexcitable period (Kiernan et al., 2020)**.**

Potassium channels, whether fast or slow, have little, if any, role in the generation of individual action potentials, as demonstrated through the negligible effects of pharmacological blockade upon action potential generation (Schwarz et al., 1995), in contrast to the effect of the sodium channel blocker, tetrodotoxin, which completely abolishes action potential generation. Instead, potassium channels act as a brake on excitability, preventing repetitive firing and contributing to maintenance of resting membrane potential (Krishnan et al., 2009; Maljevic et al., 2010; Schwarz et al., 2006)

4.4.2 Subexcitability has several determinants

The subexcitable period, measured during the recovery cycle, is a period of relative reduction in excitability, where greater current is required to achieve threshold. It has several determinants. Firstly, this phase reflects hyperpolarisation due to nodal slow potassium current through *KCNQ* channels encoding K_v7 members of the potassium channel family. A proportion of these channels are active at resting membrane potential but become increasingly activated by depolarization during impulse conduction. They are slow to deactivate, producing hyperpolarization due to outward potassium current, where greater current is required to achieve threshold (Bostock et al., 1998; Schwarz et al., 1995). Secondly it is also dependent on the difference between the resting membrane potential (E_r) and the potassium equilibrium potential (E_K) (Kiernan et al., 2000). Finally, activation of slow potassium channels by depolarisation is not manifest until the superexcitable period has decayed. Therefore, the subexcitable period is also influenced by the rate of decay of the superexcitable period preceding it. As a measure of slow K+ activity, subexcitability may be contaminated by superexcitability, and does not provide a pure measure of slow potassium current (Kiernan et al., 2020).

4.4.3 The implications of reduced subexcitability in these experiments

Based on understanding of the known determinants of subexcitability (Kiernan et al., 2020) a reduction in subexcitability could arise through 1) impairment in slow potassium channel function, 2) alteration in resting membrane potential or potassium equilibrium potential or 3) an abnormally prolonged period of superexcitability.

There is no reason to suspect that the resting membrane potential or potassium equilibrium potential of NFCI participants should be altered relative to controls. There were no additional differences between controls and the NFCI group to suggest altered membrane potential. Although electrolytes were not measured in the control participants, potassium was normal (although not necessarily measured at the same time as recording, there is no reason to suspect that it should have become abnormal) in all NFCI patients and there is no reason to suspect that it should be significantly different compared to controls. Superexcitability in the NFCI group was not significantly different from the control group. Therefore, whilst being mindful of the cautionary statement at the beginning of this discussion, a reduction in slow potassium channel function would be the most likely contributor to reduced subexcitability.

4.4.4 Naturally occurring potassium channel dysfunction as a cause of neuropathic pain is much less reported than sodium channel dysfunction

In contrast to the plethora of mutations in voltage-gated sodium channels that result in monogenic neuropathic pain states (Bennett et al., 2019), there are no potassium channel mutations that give rise to similar monogenic, inherited phenotypes. This is likely to be a major reason that there is considerably less published literature pertaining to the role of potassium channels in neuropathic pain than for sodium, or even calcium, channels (Busserolles et al., 2016) (there are, however, well characterised mutations that give rise to developmental and epileptic encephalopathies (Biervert et al., 1998; Maljevic et al., 2012), episodic ataxia Type 1 (Browne et al., 1994) and inherited neuromyotonia (Falace et al., 2007)). Neuropathic pain is experienced by patients with autoantibodies to Caspr2, a component of the voltage gated potassium channel complex, but this symptom was long

overlooked because the presenting complaints of these patients are more prominently seizures and cognitive symptoms (encephalitis) or abnormal muscle cramps and twitching (Morvan's Syndrome and neuromyotonia) (Irani et al., 2010). Nevertheless neuropathic pain has subsequently been recognised as an important feature, and it is now recognised that this may be the only feature (Binks et al., 2018). Recently it has been shown that this arises due antibody binding to Caspr2 on primary afferent (mouse) neurons (Dawes et al., 2018). Similarly, patients with painful inflammatory neuropathies have been found to have autoantibodies to Caspr1 (Doppler et al., 2016)**.** The potassium channels associated with Caspr 1 and 2, and with LGI1, however, are not slow channels and are unlikely to be of the same subfamily as those implicated in these threshold tracking experiments.

Aside from these examples the evidence for a role of potassium channels in neuropathic pain is derived almost entirely from basic science experiments and animal models, although polymorphisms can associate with an increased risk for specific types of neuropathic pain such as phantom limb pain, sciatica (Costigan et al., 2010) and also with pain intensity in HIV neuropathy (Hendry et al., 2013).

4.4.5 Experimental work supports a role for potassium channels in neuropathic pain states and cold allodynia.

As discussed in Chapter 1 several members of the potassium channel superfamily are of interest in neuropathic pain states, including fast channels, two-pore channels and slow channels (Busserolles et al., 2016; Tsantoulas & McMahon, 2014).Our knowledge of the expression patterns in the peripheral human nociceptive system is limited, but much better

understood in rodents (Figure 4.6). Furthermore, several potassium channel members have been implicated in pathological cold sensation in lab based experiments,

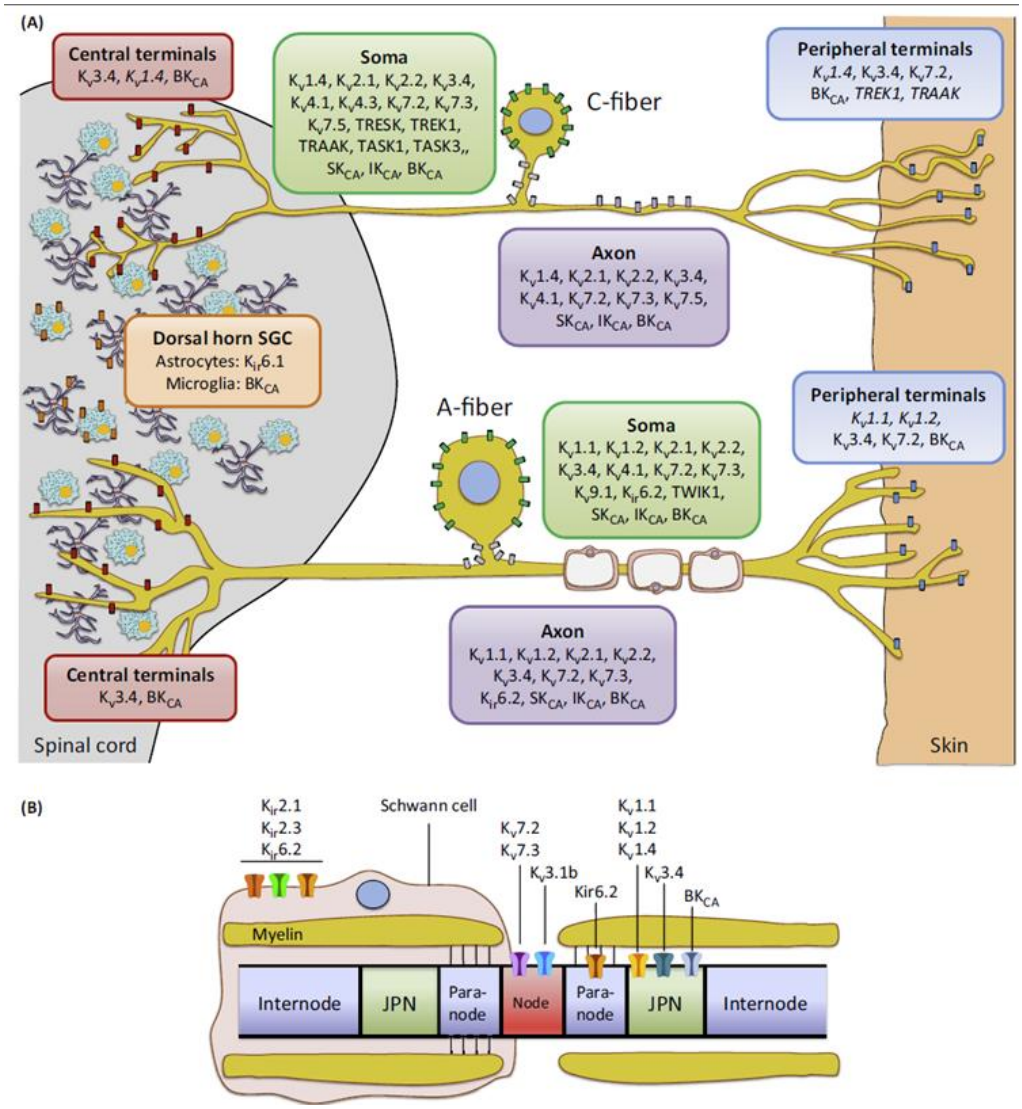


Figure 4.6 The distribution of potassium channels in the mouse peripheral nociceptive system, taken from Tsantoulas **(Tsantoulas & McMahon, 2014)**.
(A) illustrates the distribution of potassium channels throughout the peripheral nociceptive system, in unmyelinated (top) and myelinated (bottom) fibres
(B) illustrates the distribution of potassium channels along the myelinated axonal membrane and Schwann cells

including Shaker-like $K_v1.1$ and $K_v1.2$ (González et al., 2017; Madrid et al., 2009) members of the two-pore domain family (Castellanos et al., 2020; Descoeur et al., 2011; Pereira et al., 2014) and the slow channels (Abd-Elsayed et al., 2015).

4.4.6 A reduction in slow potassium current in NFCI patients with chronic pain is an attractive mechanism

The subfamily of potassium channels that mediates the slow current is the K_v7 group of channels, encoded by KCNQ genes (Schwarz et al., 2006)**.** Members of this family are expressed throughout the central and peripheral nervous systems, including the DRG (Passmore et al., 2003) the Node of Ranvier (Devaux et al., 2004) and in myelinated and unmyelinated nociceptive neurons in rodents (Du et al., 2018; King & Scherer, 2012) and humans (Lang et al., 2008; Taylor et al., 1992). Experimental data in rats suggest that KCNQ channels could modulate cold-sensitivity in pathological states (oxaliplatin neurotoxicity and chronic constriction injury). Blockade of these channels resulted in a hyperexcitable electrophysiological phenotype of dissociated trigeminal neurons. Furthermore, *in vivo*, channel blockade resulted in cold allodynic behaviour, whilst potentiation of this channel associated with an apparent amelioration in the cold allodynia caused by oxaliplatin (Abd-Elsayed et al., 2015). Retigabine, an antiepileptic drug which activates K_v7 channels, significantly reduces excitability of unmyelinated human axons *in vitro*. Moreover, in threshold tracking experiments on these axons, retigabine enhanced late subexcitability (Lang et al., 2008), the opposite to reduced late excitability observed in the NFCI patients. Retigabine also attenuates behaviours of neuropathic pain in animal models, which appears to be mediated specifically via its action on K_v7 channels (Passmore et al., 2003; Wu et al., 2017). Despite this evidence, however, this agent has performed poorly in clinical trials and

is not recommended for use in the treatment of neuropathic pain in general. It may be that if retigabine or other potassium channel activating agents were targeted more specifically to those in whom potassium currents have been shown to be abnormal, that it would show greater efficacy.

4.4.7 Abnormalities in these studies were relatively limited

In these experiments significant differences were only seen in two excitability indices, S2 accommodation in depolarising threshold electrotonus and late subexcitability in the recovery cycle, and only from motor nerve recordings of myelinated nerve fibres.

Experimentally, when potassium channels are blocked with tetraethylammonium (TEA) or with the more specific blockers of slow, KCNQ channels (XE991 and linopirdine), as well as reduction in subexcitability there is also a significant reduction in accommodation (Schwarz et al., 2006). Whilst there was a reduction in accommodation in the motor studies of the NFCI patients, this only just met significance (Table 4.2).

In patients with benign familial neonatal epilepsy due to mutations in KCNQ2 (which encodes the $K_v7.2$ subunit of the slow potassium channel), whilst a trend to reduced subexcitability has been observed, much more significant differences were observed in several other parameters, including reduced accommodation, relative refractory period and increased superexcitabilty (Tomlinson et al., 2012). Of these indices, only S2 accommodation was reduced in the NFCI group.

4.4.8 Further study is required to fully understand the basis for the changes seen in these experiments

Even if there were more extensive and significant changes to suggest altered slow potassium current, it would not be possible to explain this fully without further study. KCNQ genes were not included in the list of pertinent neuropathic pain genes used as the basis for Chapter 3, primarily because there are no reported conditions in which mutations in these genes result in neuropathic pain (one of the criteria used to curate the list of candidate genes). Future studies could analyse these genes for variants. It would also be of value to develop patient derived cell lines for electrophysiological experimentation.

4.4.9 Experiments were performed with the stimulation site maintained at 32°C

The pain in NFCI is exacerbated by coolness/cold, whereas these experiments were performed at a surface temperature of 32°C or above. Cooling during threshold tracking has been used to demonstrate changes in excitability of motor axons in healthy volunteers (Burke et al., 1999; Kiernan et al., 2001a) and in those with oxaliplatin neurotoxicity (Bennedsgaard et al., 2020). Changes in the latter study included a prolongation of relative refractory period and increased duration of sub-excitability. Although this was a very small study of only 7 patients, and cooling was not performed pre-oxaliplatin or in a control group, the findings of altered excitability at cool temperatures is clearly interesting to note in a condition where cold allodynia is such a distinctive feature. The changes in these indices would be likely to result in lower membrane excitability, overall. In healthy volunteers, cooling resulted in a prolongation of the relative refractory period, as well as slowing accommodation (Kiernan et al., 2001). Both of these studies used motor axons for excitability testing. It is difficult to extrapolate their findings to potential effects of

temperature on nociceptive activity. Kovalchuk (Kovalchuk et al., 2018) attempted excitability testing in both motor and sensory nerves of healthy volunteers, and warm and cool temperatures. The results were complicated and, disappointingly, cooling to 20°C increased refractoriness to such an extent that it did not return to baseline and measurements of relative refractory period, superexcitability and subexcitability could not be made in many cases. A significant reduction was observed in S2 accommodation, however, as seen in the NFCI patients.

4.4.10 The relevance of fibre type – motor vs sensory, large vs small

The reductions in subexcitability and S2 accommodation were only seen in motor studies, fibres for which there is no evidence of dysfunction in NFCI, on clinical grounds or in nerve conduction studies. An argument for altered potassium currents contributing to symptoms of NFCI would be more compelling were changes to be seen in sensory studies, too. The sensory fibres studied in these experiments were large fibres in mixed nerve. It may be more relevant to study small cutaneous fibres. Nevertheless, abnormalities in larger fibres may still be useful as a biomarker in painful neuropathic conditions, even if not directly reflective of small fibre pathology. Examples of the latter scenario include Fabry Disease (Geevasinga et al., 2012; Tan et al., 2005)**,** painful diabetic neuropathy (Misawa et al., 2009) and oxaliplatin induced neurotoxicity (Bennedsgaard et al., 2020). In this last publication, despite the prominent symptoms of patients being sensory (cold allodynia and tingling) changes in excitability parameters were seen only in motor nerves. Mogyoros *et al* used threshold tracking to demonstrate that cutaneous afferent nerves in patients with ALS, classically considered a pure motor disorder, were resistant to the usual changes in excitability brought about by ischaemia in healthy individuals (Mogyoros et al., 1998).

It would not be unsurprising if there were no effect of NFCI on large fibres. Nerve conduction studies were consistently normal in this cohort of NFCI patients. Nevertheless the very frequent complaints of tingling and pins and needles (dysaesthesiae) in these participants (Figure 2.7, Chapter 2), thought of as 'large fibre symptoms' (Marchettini et al., 2006; Misulis, 2012)**,** might indicate a large fibre component to their neuropathy, further suggested by the findings of impaired perception of 'large fibre modalities', such as vibration sense and joint position sense (Table 2.4, Chapter 2). With such gross abnormalities of sensory function it might be expected that greater abnormalities would be observed in these threshold tracking experiments. Potential explanations include the possibility that the dysaesthesiae are mediated by small rather than large fibres or that any large fibre excitability abnormality is more distal than the wrist. Assessment of small, unmyelinated sensory fibres by threshold tracking has proved difficult but been recently reported using a modified technique requiring specially designed pin electrodes (Hennings et al., 2017; Hoberg et al., 2019; Tigerholm et al., 2020). The most established means of examining these fibres, electrophysiologically, *in vivo*, is through microneurography, as discussed in the introduction to this thesis. This technique, however, requires considerable expertise to deploy, effectively.

4.4.11 No changes attributable to sodium channel dysfunction were observed

Although a sodium channel variant has been identified in this cohort of NFCI patients (R185H in SCN9A, as described in Chapter 3), only one of the participants harbouring the R185H patients was among those to undergo threshold tracking experiments. This individual's threshold tracking measurements were all within 95% confidence intervals of control data. No changes suggestive of sodium channel dysfunction were observed in the NFCI patients as a group. The only sodium channel isoform expressed at the human Node of Ranvier of large myelinated sensory and motor axons is thought to be $Na_v1.6$, encoded by SCN8A (Caldwell et al., 2000; Kiernan et al., 2020). The SCN9A variant described in the previous chapter may well predispose to neuropathic pain and cold allodynia, as already discussed. The channel encoded by SCN9A, $Na_v1.7$, is preferentially expressed in small nerve fibres, however (Black et al., 2012), and therefore R185H or any other variant in SCN9A, may not be expected to have any effect on large fibre excitability. This may also be the case for unidentified variants in other genes preferentially expressed in small diameter neurons.

4.4.12 Threshold tracking was only performed of the median nerve

Participants' symptoms were present in both hands and feet, as depicted in the heat map in chapter 2 (Figure 2.6). Differences in the subjective severity of symptoms between hands and feet were not captured but sensory deficits on examination were more marked in the feet than the hands (Table 2.4, Chapter 2). Perhaps abnormalities of membrane excitability would be more likely if the sural nerve was assessed. There is evidence to suggest that there are inherent differences in excitability between sural and median nerves of healthy individuals (Lin C. et al., 2000).

4.4.13 NFCI participants were not matched for ethnicity

There was difficulty recruiting individuals of African origin without symptoms of NFCI, but who had been exposed to cold. Those African controls who underwent skin biopsy were seen in Catterick, Yorkshire, and it was not feasible for the Threshold Tracking equipment to be taken there.

There are no data pertaining to any membrane excitability differences between white individuals and people of colour. This is a potential confound and should be addressed in future studies. Ethnicity dependent differences in nerve conduction studies have been observed in various population (Fong et al., 2016; Shivji et al., 2019), although no differences between black and white healthy individuals were found in one study (Buschbacher & Koch, 1999). Similarly, controls were not matched for significant cold exposures. Ideally similar control subjects would be used to those in whom intraepidermal nerve fibre density was measured in Chapter 2.

5 Discussion

5.1 Summary of results

The key findings of this thesis are as follows:

1) The late sequelae of Non Freezing Cold Injury are due to an acquired painful sensory neuropathy
2) This neuropathy is characterised by injury to small nerve fibres, demonstrated by functional and structural deficits.
3) Sensory modalities conveyed by large fibres are impaired, but in the absence of large fibre dysfunction as measured by nerve conduction studies at conventionally used sites. Several of the findings regarding phenotype have been replicated in a similar study (Anand et al., 2017), as discussed in Chapter 2.
4) The neuropathy is associated with high levels of social and psychological impairment.
5) A variant in SCN9A is disproportionately represented in this cohort of participants, occurring exclusively in African individuals.
6) When expressed in HEK293 cells, the variant bestows gain of function characteristics when studied at low temperature, providing a molecular correlate to the cold allodynia experienced by participants.
7) Nerve excitability testing suggests a reduction in slow potassium current in motor fibres, which, if also true of nociceptors, may render them hyperexcitable.

5.2 **Wider implications for the management and study of NFCI and neuropathy**.

The findings in chapter 1, that NFCI causes a painful sensory neuropathy, have already been used to inform policy in the British Armed Forces via the NFCI Independent Senior Advisory Committee (NISAC). Clinical practice has changed significantly as a result. The DN4 questionnaire is being used as a screening tool and there is a UK wide clinical service for those with NFCI. Patients now undergo neurological examination, which was not routinely the case, previously. Patients with chronic symptoms are referred to specialist centres for investigation and management of neuropathic pain. Prior to this research there was a much greater emphasis on investigation of putative vascular dysfunction, through measurements of skin blood flow or infrared thermography, for example. (personal communication Dr Sarah Hollis, Regional Occupational Health Team, Catterick Garrison, UK)

If the higher than expected incidence of R185H in *SCN9A* is confirmed in larger studies, and if relevant variants in additional genes are identified, this could provide a basis by which to risk stratify military personnel, potentially sparing them injury at all. The suggestion of reduced slow potassium current in the threshold tracking experiments provides rationale for further investigation. Whilst potassium channel activating medications have been disappointing in clinical trials, if targeted appropriately to individuals with evidence of reduced slow current, these could hold promise for the treatment of their pain.

The pattern of neuropathy in this cohort is unusual, with profound deficits in large fibre modalities, but normal nerve conduction studies. These abnormalities require further study but could represent a novel type of neuropathy characterised by very distal injury. It is quite plausible that this type of injury is present in other patients presenting with neuropathic symptoms due to other causes, particularly those cases labelled 'idiopathic'. Nerve conduction studies are frequently non-diagnostic in patients complaining of sensory loss even when clinical examination suggests impairment of larger fibres.

5.3 Wider implications for the study of neuropathy and neuropathic pain

An ethnic predisposition to NFCI has long been recognised (Burgess & Macfarlane, 2009; Orr & Fainer, 1952; Ungley, 1942). Predisposition to the inciting injury may be due to differences in regulation of skin blood flow (Iampietro et al., 1959a; Maley et al., 2014). Genetic factors such as the *SCN9A* R185H variant described in chapter 3 may increase the risk of developing chronic painful neuropathy. This thesis has highlighted the fact that there is a paucity of data pertaining to African populations in a number of areas relevant to the study of neuropathic pain. There are no published reference values for black Africans for quantitative sensory testing or intraepidermal nerve fibre densities. These are areas that require attention if large-scale phenotyping studies are to be performed. Similarly, whilst the changes seen in threshold tracking are of interest, and certainly merit further exploration, it is unknown whether or not there are inter-ethnic differences in axonal excitability.

There is greater genetic diversity and less linkage disequilibrium in African populations compared to non-African populations (Campbell & Tishkoff, 2008)**.** This is of relevance to the study of all diseases**.** Publicly available databases are inadequate for the study of subpopulations within Africa and genetic variability remains incompletely understood (Retshabile et al., 2018).

As next generation sequencing identifies increasing numbers of genetic variants interacting with environmental factors to cause neuropathic pain, experimental paradigms will need to take the environmental element into account. In chapter 3 this was achieved by performing cellular electrophysiology at low temperatures, but it may be more difficult to replicate more complex environmental factors in cell-based experiments. This may require a parallel increase in the complexity of the experimental model, at the cost of greater technical difficulty and reduced efficiency. Whilst animal models can certainly replicate this complexity to some extent, these are time consuming and costly to produce and sometimes technically difficult. Traditional rodent models are not suitable for the high throughput screening that is desirable with the accelerating identification of rare variants.

5.4 Future directions

Prospective longitudinal study (or studies) would be invaluable in understanding the pathogenesis of non-freezing cold injury, particularly in establishing those at increased risk of developing late sequelae. This would have significant implications from an occupational health perspective, especially in the armed forces. There is precedence for longitudinal studies in the neuropathic pain field (Ellis et al., 2020; Goswami et al., 2016). By recruiting participants prior to any cold exposure, it would be possible to perform assessments at

baseline and to repeat these at regular intervals during military training. Changes could be assessed in relation to cold exposures. This would also ensure that accurate data could be recorded regarding the exact nature of exposures, rather than relying on retrospective reporting. In addition, this would ensure that control subjects could be as carefully matched as possible. As well as studying those who do not develop NFCI it would be equally helpful to study those who develop NFCI but then recover. Assuming adequate funding and recruitment such a study could use all the techniques employed in this thesis and additional experiments.

5.4.1 Skin biopsy measurements

Serial measurement of intraepidermal nerve fibre density has been used to examine the structural integrity of the peripheral sensory system over time. This has been done in experimental pain models (Gibbons et al., 2010), where loss of, and subsequent recovery of IENFD following capsaicin application has been shown. Similarly, others have used serial skin biopsy to study regeneration and pattern of reinnervation following mechanical axotomy (Hahn et al., 2006; Rajan et al., 2003). The technique has also been used to demonstrate structural recovery following surgery for carpal tunnel syndrome (Baskozos et al., 2020), but also a *lack* of recovery following herpes zoster (Petersen et al., 2010).

There are striking impairments in mechanical and vibration detection in this cohort and also in a subsequent phenotyping study (Anand et al., 2017). It would be of great interest to study mechanosensory end organs in glabrous skin to investigate the possibility of a very distal injury that nerve conduction studies are insufficiently sensitive to detect. To date the only

direct evidence is from Blackwood's post mortem studies from 1944 in which Pacinian Corpuscles in one individual were seen to lack innervation (Blackwood, 1944). It would also be possible to study arteriovenous anastamoses (only present in glabrous skin (Romanovsky, 2014)), myelinated dermal nerve fibres (Provitera et al., 2007) and mechanosensory end organs (Nolano et al., 2003).

5.4.2 Assessment of psychosocial factors

The questionnaires used in this cohort revealed evidence for significant psychosocial duress. The emotional, cognitive and social components of the pain syndrome require further study. Factors of interest in this patient population include post-traumatic stress, unemployment, stigma and the roles of perceived bullying and injustice. It would be clinically valuable to explore the interdependency of these elements with chronic pain, and to identify any protective factors. This has been highlighted in a recent meta-analysis of studies exploring psychosocial factors in painful HIV neuropathy (Scott et al., 2018).

5.4.3 Threshold tracking

Hennings *et al.* have developed a technique for the selective assessment of small, cutaneous nerve fibres, and it would be interesting to apply this to patients with non freezing cold injury (Hennings et al., 2017) to see if the abnormalities suggestive of reduced slow potassium current can be replicated in a more relevant context. An important implication of this finding would be the rationale for pharmacotherapy with potassium channel activating medication. It would also be interesting to observe the effects of nerve cooling on these measures, as has

been done for oxaliplatin neuropathy (Bennedsgaard et al., 2020), particularly if such studies were performed on a longitudinal basis and correlated with patient reported symptoms.

5.4.4 Microneurography

Microneurography is a technique whereby direct recordings can be made from individual nociceptive fibres (Serra, 2012). The technique requires an expert to perform the study and is time consuming. Nevertheless even if such studies could be undertaken in only a small number of participants, this could provide useful data to help determine to what extent chronic pain, and cold allodynia in particular, are driven by peripheral processes, as has been reported in one patient with neuropathy complicated by cold allodynia (Serra et al., 2009).

5.4.5 Functional MRI

Functional MRI is an invaluable research tool in the study of pain and neuropathic pain, specifically. It is well established that the anticipation of pain can modify the way in which it is experienced, the nocebo effect. It would be of potential therapeutic utility to study this in those affected by NFCI. Functional MRI is a technique that would lend itself well to this objective and has repeatedly demonstrated activation of pain pathways in response to pain anticipation (Amanzio & Palermo, 2019; Bauch et al., 2014; Fairhurst et al., 2007; Lindheimer et al., 2019; Ploghaus et al., 1999; Porro et al., 2002). Not only has fMRI been extensively studied as a tool for measuring affective and anticipatory components of the pain experience, but there is precedence for the use of this technique to study the effects of noxious and non-noxious thermal stimuli (Brooks et al., 2002; Smith et al., 2016; Wager et al., 2013).

Simultaneous evaluation of peripheral nociceptors and brain regions with microneurography and fMRI, respectively, might permit some measure of the relative contributions of each of these regions to the overall pain experience. However, this would be extremely technically challenging.

5.4.6 Further genetic study

Many genes are implicated in neuropathic pain and cold allodynia, as discussed in chapter 3. The results of the threshold tracking experiments in chapter 4 provide a rationale to look for variants in potassium channel genes, particularly *KCNQ* genes, encoding slow potassium channels. *TRPM8* was designated a tier 3 gene in the list of pertinent neuropathic pain genes generated in chapter 3, and therefore any variants in this gene will not have been reported. Given its well established role in cold sensing and cold allodynia, this is certainly a gene of interest.

5.4.7 Elucidating the mechanisms of cold induced neural injury

There is very little evidence pertaining to how cold temperature might injure nerves. Indirect evidence suggests roles for ischaemia, impaired axonal transport, blood nerve barrier breakdown and potentially excitotoxicity. Using modern techniques it would be possible to further explore these factors. Useful systems might include skin-nerve preparation or cultured neurons, potentially co-cultured with myelinating Schwann cells (Clark et al., 2017). It would also be possible to use patient derived stem cells to ensure a relevant genetic

background. The use of Crispr gene editing technology would also allow the effects of different genetic variants to be studied in induced pluripotent stem cell lines (iPSCs). A variant of interest could be introduced to a control cell line. Similarly, if a variant was identified in a particular patient, this could be corrected. There is precedence for this approach in the study of neuropathic pain (McDermott et al., 2019). Coincidentally, this publication investigated $Na_v1.7$, the channel implicated in Chapter 3.

5.5 Conclusion

To summarise, this thesis has definitively confirmed the phenotypic and pathological nature of NFCI in the 21st Century, highlighting not only the presence of structural neural injury, but also profound psychosocial disability. The neuropathy itself is very unusual in the disconnect that is observed between loss of sensory function and normality of nerve conduction studies, a discrepancy which provides impetus for further research focussing on distal portion of the somatosensory nervous system.

Ungley's long-neglected "vaso-neuropathy" of 1942 has crossed the threshold into 21st Century research, with the implication of *SCN9A*, a giant in the neuropathic pain field. This bears comparison with Silas Weir Mitchell's "vaso-motor neurosis" (erythromelalgia) of 1872. Over nearly 150 years, our understanding of the pathology and pathogenesis of erythromelalgia has gradually evolved to a more detailed knowledge of the anatomical structures involved, and finally to multiple specific aberrations in the very same gene, *SCN9A*. NFCI was first recognised by Baron Larrey some 60 years earlier than Mitchell's description, in 1812. Perhaps, finally, it is achieving an equal footing.

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