Journal of Neurophysiology – Manuscript re-submission V6.1

Date: 25th February, 2019

Manuscript title:

Upper and lower limb motor axons demonstrate differential excitability and accommodation to strong hyperpolarising currents during induced hyperthermia.

Authors:

First author:
Oliver R Marmoy[1,2]

Second author:
Paul L Furlong[2]

Third author:
Christopher E G Moore[1]

Author affiliations:
Department of Clinical Neurophysiology, Portsmouth Hospitals NHS Trust (1)
Aston University, Birmingham (2)

Corresponding author:

Oliver R Marmoy (O.Marmoy@nhs.net). Present address is as described above as number 1 (Portsmouth Hospitals NHS Trust) and is the main affiliation address.

Author contributions:

O R Marmoy: Conception, design, acquisition, analysis, interpretation and drafting and revising of work
C E G Moore: Design, Analysis and drafting of work
P L Furlong: Design, drafting the work and revising work

All authors approve to the final version of the manuscript and are collectively accountable for all aspects of the work. All persons designated as authors qualify for authorship.

Acknowledgments:

Oliver Marmoy thanks participants for their kind donation of time.

Key points summary:

- We have used peripheral nerve axonal excitability studies to investigate the physiological differences in nerves of the upper and lower limb at rest and during hyperthermia.
- This study presents novel data to demonstrate that the response of nerves, *the accommodation,* to marked hyperpolarisation is greater in the lower limbs in healthy participants. This indicates an increase in I_H current and implies a difference in the function or expression of inward rectifying HCN channels between the upper and lower limbs. We found additional data to suggest increased nodal Na^+ current and decreased slow K^+ conductance in tibial nerve axons.The difference in HCN channel function in the lower limbs may contribute to a reduction of sustainable axon firing and thus contribute to the earlier neurological symptoms seen in distal-symmetric polyneuropathies.
- These findings encourage study of long-strong hyperpolarising currents in sensory axons and in patients with early or established distal symmetric polyneuropathy, such as those associated with diabetes or systemic disease.

Key words: *Peripheral; Neuropathy; HCN channels; Axon; Hyperthermia; Hyperpolarisation; Nerve excitability*

Abbreviations:

APB: Abductor Pollicis Brevis
AH: Abductor HallucisHCN: Hyperpolarisation-activated cyclic nucleotide-gated I/V: Current-threshold
CAMP: Cyclic Adenosine Monophosphate
K^+: Potassium
LDPN: Length Dependent peripheral neuropathy
LSP: Late Sub-excitable Period
Na^+: Sodium
NHS: National Health Service
NIHR: National Institute for Health Research
NSHCS: National School of Healthcare Science
RC: Recovery Cycle
RP/RRP: Refractory Period/Relative Refractory period
SD: Strength-duration
SNP: Super-normal period
SR: Stimulus response
TE: Threshold Electrotonus
TEh/TEd: Hyperpolarising/depolarising threshold electrotonus

Funding:

This research was financially supported from Health Education England through the NSHCS Scientist Training Programme funding stream and was registered on the NIHR portfolio (Ref. 193691).

Competing interests:

No conflict of interest to disclose.

Abstract:

Length-dependent peripheral neuropathy typically involves the insidious onset of sensory loss in the lower limbs before later progressing proximally. Recent evidence proposes HCN channels as dysfunctional in rodent models of peripheral neuropathy, and therefore differential expression of HCN channels in the lower limbs was hypothesised as a pathophysiological mechanism accounting for the pattern of symptomatology within this study. We studied six healthy participants using motor axon excitability including strong and long (-70% & -100% TEh) hyperpolarising currents to preferably study HCN channel function from the median and tibial nerves from high (40%) and low (20%) threshold. This was recorded at normothermia (~32°c) then repeated during hyperthermia (~40°c) as an artificial hyperpolarising axon stress. Significant differences between recovery cycle, superexcitability, accommodation to small depolarising currents and alterations in late stages of the inward rectifying currents of strongest (-70 and -100% TEh) currents were observed in the lower limbs during hyperthermia. We demonstrate differences in late I_H current flow, which implies higher expression of HCN channel isoforms. The findings also indicate their potential inference in the symptomatology of length-dependent peripheral neuropathies and may be a unique target for minimising symptomatology and pathogenesis in acquired disease.

New and noteworthy:

This study demonstrates nerve excitability differences between the upper and lower limbs during hyperthermia during hyperthermia, an experimentally induced axonal stress. The findings indicate that there is differential expression of slow HCN channel isoforms between the upper and lower limb, which was demonstrated through strong, long hyperpolarising currents during hyperthermia. Such mechanisms may underly postural control but render the lower limbs susceptible to dysfunction in disease states.

1. Introduction:

Upper and lower limb nerve axons differ in their functional properties and demands as afferent and efferent sensorimotor pathways. This difference may result from primary or

secondary neurological development due to length-dependent factors, but is poorly understood. It is known that in diseased states, such as in uremic, diabetic or chemotherapy-induced neuropathy, neuropathic symptoms predominate in the lower limbs and is therefore termed 'Length-dependent peripheral neuropathy' LDPN. This term of LDPN describes this common form of peripheral neuropathy where symptoms typically begin with sensory disturbance in the lower limb as tingling, hypoesthesia and neuropathic pain, but is one of the most common comorbidities worldwide and significantly contributes to chronic pain, foot ulceration and below knee amputation (Boulton, 2005) In time, there is progression proximally and later upper limb involvement (Dyck, et. al., 1985; Said, 2007). The length-dependent difference of upper and lower limbs may contribute to the pathogenesis responsible for the distal generation of symptoms and may be a result from direct hyperglycemic damage, poorer axoplasmic flow and vascular supply, increase in neurotoxicity, decreased metabolite availability or axon re-growth inhibition (Low, Lagerlund, & McManis, 1989; Arnold, Kwai, & Krishnan, 2013).. There is, however, no single mechanism to explain the predisposition of the lower limb to earlier dysfunction as seen in LDPN (Stevens, Feldman, & Greene, 1995; Quasthoff, 1996; Tavaoli, et. al., 2008). Recent evidence suggests that Hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels in rodents are pathologically modified in acquired disease states and may therefore contribute to the symptomatology seen in LDPN (Tu, et.al., 2010; Tsantoulas, et al., 2017). The HCN channels are responsible for generating a small depolarisation of the axonal membrane, through the I_H current, in response to hyperpolarisation. They are unique in being the only nerve ion channel to be activated by strong hyperpolarisation and serve to bring a hyperpolarised membrane back to the resting membrane potential before further activation. Evidence to demonstrate a biophysical difference between upper and lower limb axons which predisposes them to earlier dysfunction and symptomatolohy in healthy individuals however, is scant (Krishnan, Lin, & Kiernan, 2004).

Nerve excitability studies are now an established technique for the study of membrane and nodal properties of an axon, using alterations in pre-stimulus conditioning currents or temporal characteristics of the stimulus to track changes in a determined target response (Bostock, Cikurel, & Burke, 1998; Kiernan, et. al., 2000). Five measures of excitability are typically recorded, including the Stimulus-response (SR), Strength-Duration (SD), Current-Threshold (I/V), Threshold Electrotonus (TE) and Recovery Cycle (RC); details of these recording indices are detailed in the review by Kiernan, Burke, Andersen, & Bostock (2000). These methods have previously employed ischemia as a nerve stressor to show that slow K^+ conductance is better expressed in the upper limb (Krishnan, Lin, & Kiernan, 2004; Kirshnan, Cindy, & Kiernan, 2005). Within-nerve length dependence of the peroneal nerve corroborated these findings (Kuwabara, et al., 2000; Kuwabara, et. al., 2001). Temperature stress of the axon at the point of stimulus showed differential changes in excitability, between fibres of different activation thresholds and corroborated the theory of higher HCN channels in low threshold fibres (Moore, Cockerill, & Marmoy, 2016; Trevillion, 2010). The

differential effect of marked hyperpolarisation and hyperthermia on HCN channel function between the upper and lower limbs has, however, not yet been studied (Burke, et. al., 1999; Kiernan, Cikurel, & Bostock, 2001).

Experimental excitability in rodents suggest that HCN channel function is compromised in acquired neuropathy states (Tu, et.al., 2010; Tsantoulas, et al., 2017). This is consistent with early studies showing greater inward rectification in diabetic patients (Shimantani, et. al., 2015; Horn et. al., 1996). The contribution of HCN channels within human LDPN has not been studied, but firstly the particular distribution of HCN channels between the upper and lower limbs is not known, nor how HCN channels may differ in response to physiological stress such as hyperthermia, which is examined in this study. This may lower the safety margin for activation and be a likely mechanism of HCN channels inability to counteract the effects of axon hyperpolarisation induced through hyperthermia. It has been demonstrated that HCN2 isoforms significantly contribute to neuropathic pain, also seen in animal models of acquired neuropathies and would indicate their potential pathogenesis (Emery, et. al., 2012; Young, et. al., 2014 ;Tsantoulas, et al., 2017;). Therefore, due to its interest in this study, hyperthermia was chosen to induce functional hyperpolarising changes in axons of the upper and lower limb to identify differences between HCN channel expression or activation contributes to the earlier dysfunction in LDPN. Low-threshold fibres (20%) were particularly targeted due to their known increased I_H current and to improve participant tolerance during lower limb stimulation (Trevillion, et. al., 2010).

The aim of this study was to identify functional differences in nerve excitability between the upper and lower limbs, particularly with regard to HCN channels, in healthy individuals. We hypothesised that there may be different physiological mechanisms between the upper and lower limbs which may render the lower limbs more susceptible to dysfunction in disease states.

3. Methods:

Ethical approval:

All participants provided written informed consent with a favourable ethical opinion given from the study sponsor (Aston university), with local NHS research and innovation approval provided by the host organisation (Portsmouth Hospitals) before registering on the NIHR portfolio database. The study conformed to the standards set in the Declaration of Helsinki except for registration in a database.

Nine healthy participants were studied with six qualifying to data analysis (age range 18-49, M=F). The three participants excluded were due to poor stimulus tolerance (N=2) and asymptomatic Carpal Tunnel Syndrome (N=1). Nerve excitability was stimulated and recorded at two sites; first stimulation and recording was performed at the wrist with the stimulating cathode at the distal wrist crease of the dominant hand and the anode placed

proximally on the forearm, with the Compound Muscle Action Potential (CMAP) recorded from the Abductor Pollicis Brevis (APB) of the thenar eminence. Second stimulation and recording was performed with the stimulating cathode at the ankle (1-2cm posterior and inferior to the medial malleolus) over the course of the tibial nerve, with the anode placed 8-10cm proximally over the bony protrusion of the tibia, with the active recording electrode placed over the Abductor Hallucis (AH) muscle with the reference electrode placed 6cm distally over the bony metatarsophalangeal joint (Figure 1). Stimulation at the ankle required compression with a small plastic cylinder to ensure effective stimulation of the tibial nerve. Ground electrode was placed over the dorsum of the hand or foot respectively.

Excitability indices of Stimulus Response (SR), Strength Duration (SD), Threshold Electrotonus (TE), Current-threshold (I/V) curve and the Refractory Period (RP), on high (40%) or low (20%) threshold fibres were generated (Bostock, Cikurel, & Burke, 1998). Strength duration was sampled at stimulus widths of 0.2, 0.4, 0.6, 0.8 and 1ms, threshold electrotonus recorded to conditions of +20%, +40%, -20%, -40%, -70% and -100%. Stimulation and recording were automatically controlled via a PC running the QTRAC nerve excitability programme and a subroutine named TRONDNF which records the five excitability indices listed above (Bostock, Cikurel, & Burke, 1998). Stimuli were delivered via a Digitimer DS5 (Digitimer Ltd., Welwyn Garden City, UK) with a Grass LP511 AC Amplifier (Grass Technologies, Natus Neurology ©, Rhode Island, USA) used to amplify the CMAP with bandpass filters (2Hz – 10kHz) before digitised with a sampling rate of 20kHz.The HumBug (Digitimer Ltd., Welwyn Garden City, UK) was used to remove 50Hz noise prior to digitising the signal through the analogue to digital converter (National Instruments PCI-6221, NI Corporation, Austin, USA). Skin was prepared using abrasive gel (NuPrep, Weaver and Company, Colorado, USA) and an alcohol wipe to minimise skin impedance. Disposable sticky electrocardiographic electrodes were attached to deliver the stimulus current to the desired nerve (Ambu ® BlueSensor ECG electrodes, Ambu, Denmark) with small polarisable sticky recording electrodes used for recording the CMAP (Ambu ® Neuroline Surface electrodes, Ambu, Denmark).

The procedure followed a protocol whereby ‘high’ (40%) and ‘low’ (20%) target thresholds were measured first during normothermia from the median and tibial nerves respectively. The benefit of hyperthermia as an artificial stress was demonstrated by Howells et. al. (2013) where it was found that slow K^+ channels ‘dampen’ the excitability of axons and more importantly, I_H current becomes more hyperpolarised in hyperthermia. A minimum of 15 minutes between recording periods was given to minimise effects of activity dependent hyperpolarisation (Kuwabara, et. al., 2002). After which, the arm was placed within a polythene sheath and submerged under a temperature-controlled water bath at 41-42°c for a minimum of 20 minutes until the limb temperature remained stable for >5 minutes to ensure the nerve was adequately heated. During pilot studies a rise in systemic temperature was seen following limb warming, so to minimise confounding temperature variables normothermic measurements were all made prior to warming of any limb. The water bath

was installed with a custom-made material sling to improve comfort and avoid blockage of water turbulence. Temperature was recorded using a thermometer calibrated against a thermistor thermometer accurate to 0.1°c, with a cardboard housing placed over the probe to ensure this only recorded skin temperature not external temperature (Drager^{TM} HNICU-36, DeRoyal Industries Inc., USA). Nerve excitability was repeated during hyperthermia with low threshold fibres. The experimental protocol then repeated followed for the tibial nerve at the ankle. Throughout testing a minimum of five minutes between recordings was implemented to avoid any activity dependent axon hyperpolarisation (Kuwabara, et. al., 2001).

All patients were tested using traditional nerve conduction studies and a screening questionnaire to exclude a history of neurological or diabetic disease. Data collected was tested for normality using the Lillifors test, using paired student t-tests on all recorded variables between study groups of limb and temperature, following a Benjamini-Hochberg procedure with a false discovery rate of 10% to correct for multiple comparison measures. Thus, P-value was dependent on its variable rank size and number of comparisons made.

FIGURE 1 HERE

4. Results:

Nerve excitability was recorded at high (40%) and low (20%) threshold fibres of the median and tibial nerves during normothermia and from low threshold fibres from the median and tibial nerves during hyperthermia. The mean resting, hyperthermic and temperature differences during each scenario are seen in Table 1.

NERVE TESTED	NORMOTHERMIC TEMPERATURE (°C) OF 40% + 20% THRESHOLD FIBRES	HYPERTHERMIC TEMPERATURE (°C) OF 20% THRESHOLD FIBRES	MEAN INCREASE (°C)
MEDIAN (WRIST)	31.7 ± 0.5	39.3 ± 0.2	7.6 ± 0.6 (P=0.0032)
TIBIAL (ANKLE)	29.1 ± 0.5	39.3 ± 0.2	10.5 ± 0.6 (P=0.0032)

Table 1 – Mean temperature recordings and change during normothermia and during induced hyperthermia using the thermostatically controlled water bath. All measurements were made using a thermistor calibrated thermometer recording skin temperature within 3cm of the stimulating cathode. Insulative shielding to minimise the confounding influence of external heat on recorded temperatures was ensured.

CMAP latency recorded from APB during median nerve stimulation significantly decreased by 0.94ms ± 0.1ms during hyperthermia (P=0.0194). A non-significant small reduction of 4.4% was seen in the peak CMAP amplitude and increase of 15.9% in the stimulus required to elicit 50% of the peak response in hyperthermia. CMAP latency recorded from AH during tibial nerve stimulation reduced significantly during hyperthermia by 1.63ms ± 0.2ms (P=0.0004). A significant reduction of 15.9% in the peak CMAP was seen during

hyperthermia (P=0.004) alongside a non-significant decrease in the stimulus required to elicit 50% of the peak response. Therefore these changes were not thought to reflect any significant conduction block during hyperthermia, as all peak amplitudes remained within 20% of their normothermic measures.

4.1 Changes in strength-duration (SD)

Strength duration properties were not significantly different between temperature variables in either median or tibial nerves (Figure 2). However, axon Rheobase exhibited a larger mean change of 1.1mA in the lower limb compared with a mean change of 0.3mA in the upper limb, seen as a slope change of strength-duration indicating less threshold change to longer stimuli in the lower limb during hyperthermia.

FIGURE 2 HERE

4.2 Changes in recovery cycle (RC)

The recovery cycle in the median nerve demonstrated significant reduction in the Relative Refractory Period (RRP) of 0.484ms (P=0.019), Super-normal Period (SNP) (P=0.0419; including at 5 and 7ms) and refractoriness at 2.5ms (P=0.0258) during hyperthermia (Figure 3A). A non-significant reduction in LSP area of 3.6% ± 1.6% was observed during hyperthermia. Tibial nerve recovery cycle showed only a significant reduction in refractoriness at 2.5ms (P=0.0226) during hyperthermia (Figure 3B). Comparisons between limbs in hyperthermia showed the tibial nerve to have significantly larger SNP (P=0.0125) with no significant differences in RRP or LSP.

FIGURE 3 HERE

4.3 Changes in Threshold Electrotonus (TE)

4.3.1 Depolarising currents (TEd)

Threshold electrotonus to small depolarising currents in the median nerve (Figure 4A) demonstrated significant decreases in TEd 10-20ms (P=0.0387), TEd peak (P=0.0161), TEd undershoot (P=0.0226) alongside S2 accommodation (P=0.0022) and accommodation half time (P=0.0032) during hyperthermia. The findings were less significant in the tibial nerve during hyperthermia where only TEd 40-60ms (P=0.0129) and accommodation half-time (P=0.0097) were significantly decreased (Figure 4B). Comparisons between different nerves during hyperthermia demonstrated a significantly larger

TEd (40-60ms) and S2 accommodation half-time in the hyperthermic tibial nerve (upper red arrow, Figure 4C).

FIGURE 4 HERE

4.3.2 Hyperpolarising currents (TEh)

Small hyperpolarising subthreshold currents during hyperthermia demonstrated a significant decrease in the TEh overshoot of both median and tibial nerves compared to normothermia (P<0.01). The accommodation to strong and long -70% (200ms) and -100%

(300ms) hyperpolarising currents to the median nerve showed a significant decrease in the S3 for -70% (P=0.0452) and S3 for -100% (P=0.0065), with just significant differences in the TEh 101-140ms slope (P=0.0516), all of which consistent with the graphical TE findings (Figure 4A) Whilst accommodation to strong and long hyperpolarising currents to the tibial nerve failed to demonstrate a significant change, some speculation is made to the similar graphical trend of S3 in Figure 4B being similar to Figure 4A of the median nerve. Threshold electrotonus to strong and longer hyperpolarising currents of different nerves during hyperthermia showed significantly less threshold reduction in the strongest and longest (-100%, 300ms) hyperpolarising current S3 phase (lower red arrow, Figure 4C). No significant differences were seen in TEh peaks of -70% and -100% curves.

4.4 Changes in current-threshold (I/V) relationship

There was no difference between median and tibial nerves at rest and no statistically significant change in either nerves on heating (Figure 5).

FIGURE 5 HERE

5. Discussion:

Axonal excitability studies in lower limb motor axons have shown that hyperthermia has marked effects on recovery cycle super-excitability and accommodation to small depolarising currents. Also, and previously unreported, we demonstrate late onset differences in the response to strong hyperpolarising currents between the upper and lower limbs during hyperthermia. This demonstrates differential HCN channel behaviour between the upper and lower limbs which, as recent evidence suggests, may render the lower limbs more susceptible to dysfunction in disease states and could underly the pathophysiological mechanisms responsible for the symptomatology seen in LDPN.

5.1 Effects of hyperthermia on stimulus response, strength-duration and recovery cycle between limbs

Hyperthermia demonstrated opposing shifts of the stimulus response compared to normothermia; the median nerve necessitated an increased stimulus to elicit a peak response, whereas the tibial nerve reduced the stimulus required. There was nothing to suggest temperature-related changes at the neuro-muscular junction or directly to muscle fibres; maximum amplitude and morphology of the CMAP remained relatively stable and there was no evidence of conduction block. Therefore this change can be taken to show that the median nerve axons at the wrist were more hyperpolarised, and therefore required a higher stimulus current to exceed the axon threshold (Henderson et. al., 2006). This is supported by the reduced strength-duration slope, alongside a slight reduction in the raw peak CMAP amplitude, constituting a level of local axon hyperpolarisation (Krishnan, et. al., 2009). Such changes indicate that the tibial nerve may be better able to counteract chronic hyperpolarising changes compared to the median nerve.

Hyperthermia in both the upper and lower limb axons from resting temperature demonstrated reductions in refractoriness, with reduction in RRP also seen in the upper

limb. This suggests an acceleration of fast K^+ channel kinetics in the node and paranode caused by hyperthermia of the axon, as the opposite effect is seen during cooling (Kiernan, Cikurel, & Bostock, 2001). Comparisons between median and tibial nerves during hyperthermia revealed no significant differences. This suggests that fast K^+ kinetics are not significantly different between the upper and lower limbs and is consistent with previous findings in the peroneal nerve (Kuwabara et. al., 2000). Overall, this left-ward shift of the RC curve again indicates hyperpolarisation of the axon membrane (David et. al., 1995).

5.2 Increased nodal Na+ driving current in tibial motor axons

A significant reduction of median nerve supernormality (SNP) was seen in hyperthermia. This was surprisingly not seen in the tibial nerve despite a larger temperature change from rest which would have been predicted (Burke et. al., 1999). The SNP originates from a depolarising after-potential through spontaneous charge flow into the node from current stored in the internode. This makes the SNP very sensitive to internodal resistance properties (David et. al., 1995; Kiernan & Bostock, 2000). Whilst the axon hyperpolarisation could create differences in the SNP, this would be a result of extra-axonal K^+ accumulation due to activation of the Na^+/K^+ pump, which would cause significant differences in RRP between limbs, a finding not seen previously (Kuwabara et. al., 2001). It could be possible that the larger and resilient SNP of the tibial nerve may reflect greater capacitance in the internode creating a larger SNP, however this is physiologically implausible. A longer internodal region should proportionally increase conduction velocity, but tibial nerve velocity is slower than the median nerve (Simpson, et al., 2013; Kimura, 2013). In addition, differences in axon diameter would cause disparity in the RRP which was not seen between nerves (Kiernan et. al., 2000). It is therefore suggested that these findings are attributed to an increased nodal Na^+ driving current to overcome hyperpolarisation and incur a larger SNP as seen in the tibial nerve. An alternate hypothesis would be an increased myelin thickness though this is not supported by histological findings (Debanne et. al., 2011).

5.3 Decreased slow K+ conductance in tibial nerve axons

During hyperthermia the TEd 40-60ms, representing the S2 phase, was significantly increased in the tibial nerve compared to the median nerve. The S2 period reflects a decrease in excitability of the axon, physiologically attributed to the activation of slow nodal K^+ channels (Kiernan et. al., 2000). This was supported by a significant increase in the accommodation half-time of tibial axons, indicating a longer accommodation of slow K^+ channels to restore membrane potential (Bostock & Baker, 1988). The TEd overshoot phase, attributed to deactivation of slow K^+ channels, was not significantly different between upper and lower limbs in hyperthermia. This finding indicates that although the kinetics of the slow K^+ channels are similar between upper and lower limbs, the slow K^+ channel expression is reduced in the lower limb. This difference in physiology gives one mechanism which may render the lower limbs more susceptible to dysfunction in disease conditions (see also; Kuwabara et. al., 2000; Kuwabara et. al., 2001).

5.4 Differences in hyperpolarising threshold electrotonus: Evidence for different HCN channel expression of the tibial nerve

A significant reduction in TEh overshoot was seen in both axons during hyperthermia when compared to normal temperature. As TEh is proportionate to deactivation of slow K^+

channels and deactivation of I_H current, these findings would support the findings in TEd of less expressed slow K^+ channels in the lower limb.

Perhaps most novel of the findings was a significantly increased S3 period to the strongest and longest (-100% for 300ms) hyperpolarising currents of the tibial nerve compared to the median nerve in hyperthermia. This demonstrates greater accommodation in the tibial nerve during hyperthermia, which implies a greater inward rectifying I_H current in the tibial nerve relative to the median nerve. This study was unable to directly compare differences of I_H current at rest due to differences in resting temperatures of the two nerves. Previous studies found no significant differences between S3 phases (Kuwabara, et. al., 2001). It is therefore implied that when subjected to hyperthermic stress, the lower limb tibial nerve exhibits greater accommodation to hyperpolarisation through higher I_H current, probably due to increased conductance of individual channels although this has not been studied in vitro (Tomlinson et. al., 2010). We therefore speculated that HCN channels are expressed differently in the lower limb or the HCN channels themselves have different properties that permits greater I_H current conductance.

Whilst different HCN channel expression is likely to be the rationale of these findings, the graphical changes indicate a mechanism unanticipated by the original hypothesis. It was surprising that the most significant change was mostly notably after 200ms of TEh -100% plots. This is rather atypical for previous studies of HCN excitability in the median nerve due to its late onset and demonstrates why these changes are not reflected significantly within the I/V curve due to its shorter stimulus duration (Tomlinson et al., 2010; Howells et. al., 2012). Of the four HCN isoforms both HCN1 and HCN2 are known to be distributed in human peripheral nerve, but HCN3 is of uncertain distribution and HCN4 primarily distributed in the central nervous system (Doan et. al., 2004). Whilst HCN1 was originally hypothesised to be expressed differently in the lower limb, these findings allow theory that a slower HCN isoform is a more likely to be responsible for these late changes due to its time of activation. It has been documented in cellular studies that the activation times for HCN1-4 are 30ms, 184ms, 265ms and 461ms respectively, which therefore makes HCN2 and HCN3 viable candidates and also supports the theory that slower HCN isoforms underpin the late strong hyperpolarisation as postulated by Nodera and Rutkove (2012) and Howells et. al. (2012). As no data exists on HCN isoform location in peripheral axons of humans in vitro, it may be that our observations represent the first indication of the existence of slower HCN isoforms in human peripheral nerves.

6. Dysfunctional HCN channels: Potential implications for LDPN

HCN channel dysfunction has been demonstrated in experimentally induced diabetic wild-type mice or rat nodose ganglion cells (Tu et. al., 2010; Shiimantani, et al., 2015). There is little study of HCN channel function, through additional hyperpolarising protocols, in early or established acquired neuropathy in humans *in vivo*. Research of which now appears necessary to establish whether our speculations of this mechanism has a substantial contribution to the development to the distally generated symptoms in actual patients with LDPN. Studies in sensory axons may further elucidate a mechanism of a slow HCN channel

isoforms, especially given the higher expression of HCN channels in sensory nerve axons (Howells et al., 2012).

We speculate that a difference in HCN channel expression in the lower limbs seen in these findings would have direct implications for the symptomatology of acquired neuropathies. Previous studies in diabetic neuropathy have demonstrated subtle inward rectification which indicates altered I_H current (Horn et. al., 1996). Limb paraesthesia and neuropathic pain can be the most common symptoms in acquired neuropathy, which is a pertinent feature due to the mechanism of paraesthesia being a result of ectopic impulse activity where axons activate and discharge asynchronously or spontaneously (Dyck et. al., 1985; Mogyoros, Bostock, & Burke, 2000). Where repetitive firing may occur, such as in trains of impulses, axons are known to hyperpolarise (Bostock & Bergmans, 1994; Kiernan et. al., 1997). Therefore, an inability to reverse a hyperpolarised axon state effectively may lead to the development of ectopic impulse activity and worsen paraesthesia by reducing the safety margin for conduction (Kiernan et. al., 1997). Relative overactivity of mechanisms of hyperpolarisation during pathological stress would therefore render a nerve more excitable and liable to generate action potentials. This is a known mechanism of Cyclic Adenosine Monophosphate (CAMP) and temperature dependent tachycardia and may also explain the worsening of neuropathic symptoms when hot. HCN2 channels also play an important contribution to the generation of neuropathic pain seen in mouse models of acquired neuropathy (Jiang et. al., 2008; Tsantoulas, et al., 2017), whilst their inhibition with Ivabradine and Lamotrigine, an anti-anginal and anti-epileptic medication, have HCN blocking properties and are interestingly both clinically effective in neuropathic pain relief (Eisenberg et. al., 2001; Young, et. al., 2014). Both these medications drive functional kinetic differences in HCN channel function and thus future research using the methods described within this study may help elucidate their mechanism of action in future *in-vivo* studies of patients with LDPN.

7. Why would late HCN channels be more expressed in the lower limbs?

From a physiological perspective, the justification behind differential HCN channel expression is uncertain. Based on the Henneman size principle, slower motor fibres are recruited earlier, thus it is possible that the changes in I_H current may reflect differences in slow and fast axons. As AH had a higher stimulus requirement in order to elicit a peak response, it is feasible that more 'slow' fibres were recruited, contributing to the findings (Mendell, 2005; Lorenz & Jones, 2014; Kudina & Andreeva, 2014). It is, however, our theory that the lower limbs require more stable tetanic contraction to maintain weight bearing and posture control, therefore their greater firing rates and need to counter excessive hyperpolarisation is managed through this proposed upregulation or higher expression of HCN channels (Johns & Fuglevand, 2011). This would be supported by the fatigue resistance of the AH muscle, meaning a slower HCN isoform may be in higher concentration to limit hyperpolarisation and permit more continuous firing of axons (Kelly, Racinais, & Cresswell, 2013). Such hypotheses could be supported by similar experimental methodology in quadrupeds. This physiological compensation for more stable firing required for postural control but therefore may render the lower limbs more susceptible in disease states.

Conclusions

This study is the first known data demonstrating the differences in axonal excitability of the lower limb during hyperthermia. It was found that there is most likely greater inward nodal Na^+ driving current and poorer expression of slow K^+ conductance in the lower limbs. We have discovered novel findings of differential accommodation to strong and long (-100%, 300ms) hyperpolarising currents during hyperthermia between the upper and lower limbs which demonstrated greater I_H conductance in the lower limb tibial nerve. The late onset of this change is speculated to be a result of a slower HCN channel isoform, possibly HCN2 or HCN3. The findings of this study suggest that the differential expression of HCN channels between upper and lower limbs may contribute to the patterns of symptoms in some diseases as a potentially new liable site for dysfunction in acquired neuropathy. Further studies are necessary to confirm this last speculation, particularly with investigation of sensory axons and in diabetic patients without neuropathy, to identify whether this technique may be of use in early diagnosis of neuropathy where traditional nerve conduction studies lack sensitivity. Positive findings may prompt later experimental models may be beneficial to establish a pharmacological role of HCN channels in the early diagnosis of LDPN and management of disease progression and pain relief. The ability to imply ion channel function and dysfunction in humans in vivo using the methods of nerve excitability should also help take research directly to the bedside and reduce the number of animal studies needed in the development of new treatments.

References

Bostock, H., & Baker, M. (1988). Evidence for two types of potassium channel in human motor axons in vivo. *Brain Research, Volume 462*, 354-359.

Bostock, H., Cikurel, K., & Burke, D. (1998). Threshold tracking techniques in the study of human peripheral nerve. *Muscle and Nerve, Volume 21, Issue 2*, 137-158.

Boulton, A. J. (2005). Management of diabetic peripheral neuropathy. *Clinical Diabetes, Volume 23*, 9-15.

Burke, D., Mogyoros, I., Vagg, R., & Kiernan, M. C. (1999). Temperature dependence of excitability indices of human cutaneous afferents. *Muscle & Nerve, Vol. 22*, 51-60.

Dyck, P. J., Karnes, J. L., Daube, J., O'Brien, P., & Service, F. J. (1985). Clinical and neuropathological criteria for the diagnosis and staging of diabetic polyneuropathy. *Brain, Volume 108*, 861-880.

Emery, E. C., Young, G. T., & McNaughton, P. A. (2012). HCN2 ion channels: an emerging role as the pacemakers of pain. *Trends in Pharmacological Sciences, Volume 33,* 456-463.

499 Howells, J., Trevillion, L., Bostock, H., & Burke, D. (2012). The voltage dependence of Ih in
500 human myelinated axons. *Journal of Physiology, Volume 590*, 1625-1640.

501 Howells, J., Czesnik, D., Trevillion, L., & Burke, D. (2013) Excitability and the safety margin in
502 human axons during hyperthermia. *Journal of Physiology, Volume 591,* Issue 12,
503 3063-3080.

504 Jiang, Y. Q., Sun, Q., Tu, H. Y., & Wan, Y. (2008). Characteristics of HCN channels and their
505 participation in neuropathic pain. *Neurochemical Research, Volume 33*, 1979-1989.

506 Johns, R. K., & Fuglevand, A. J. (2011). Number of Motor Units in Human Abductor Hallucis.
507 *Muscle & Nerve, Volume 43, Issue 6*, 895-896.

508 Kelly, L. A., Racinais, S., & Cresswell, A. G. (2013). Discharge properties of abductor hallucis
509 before, during, and after an isometric fatigue task. *Journal of Neurophysiology,*
510 *Volume 110, No. 4*, 891-898.

511 Kiernan, M. C., Burke, D., Andersen, K. V., & Bostock, H. (2000). Mulitple measures of axonal
512 excitability: A new approach in clinical testing. *Muscle and Nerve, Volume 23, Issue 3*,
513 399-409.

514 Kiernan, M. C., Cikurel, K., & Bostock, H. (2001). Effects of temperature on the excitability
515 properties of human motor axons. *Brain, Volume 124*, 816-825.

516 Krishnan, A. V., Lin, C. S.-Y., & Kiernan, M. C. (2004). Nerve excitability properties in lower-
517 limb motor axons: evidence for a length-dependent gradient. *Muscle and Nerve,*
518 *Volume 29*, 645-655.

519 Krishnan, A. V., Lin, C. S.-Y., Park, S. B., & Kiernan, M. C. (2009). Axonal ion channels from
520 bench to bedside: A translational neuroscience perspective. *Progress in*
521 *Neurobiology, Volume 89, Issue 3*, 288-313.

522 Kuwabara, S., Cappelen-Smith, C., S-Y, L. C., Mogyoros, I., & Burke, D. (2001). Differences in
523 accomodative properties of median and peroneal motor axons. *Journal of Neurology,*
524 *Neurosurgery and Psychiatry, Volume 70*, 372-376.

525 Kuwabara, S., Cappelen-Smith, C., S-Y, L. C., Mogyoros, I., Bostock, H., & Burke, D. (2000).
526 Excitability properties of median and peroneal motor axons. *Muscle & Nerve, Volume*
527 *23*, 1365-1373.

528 Kuwabara, S., Cappelen-Smith, C., Lin, C. S., Mogyoros, I., & Burke, D. (2002). Effects of
529 voluntary activity on the excitability of motor axons in the peroneal nerve. *Muscle &*
530 *Nerve, Volume 25, Issue 2*, 176-184.

531

Kuwabara, S., Lin, C. S., Mogyoros, I., Cappelen-Smith, C., & Burke, D. (2001). Voluntary contraction impairs the refractory period of transmission in healthy human axons. *The Journal of Physiology, Volume 531, Part 1*, 265-275.

Mogyoros, I., Bostock, H., & Burke, D. (2000). Mechanisms of paresthesias arising from healthy axons. *Muscle & Nerve, Volume 23*, 310-320.

Moore, C. E. G., Cockerill, H. J., & Marmoy, O. R. (2016). Temperature dependent changes in HCN channel current (IH) in human motor axons of different threshold. Implications for generation of febrile seizures. *Clinical Neurophysiology, Volume 127,* e18-e132.

Nodera, H., & Rutkove, S. B. (2012). Accomodation to hyperpolarising currents: Differences between motor and sensory nerves in mice. *Neuroscience Letters, Volume 518, Issue 2*, 111-116.

Said, G. (2007). Diabetic neuropathy - a review. *Nature: Clinical practice - Neurology, Volume 3*, 331-340.

Trevillion, L., Howells, J., Bostock, H., & Burke, D. (2010). Properties of low-threshold motor axons in the human median nerve. *Journal of physiology, Volume 13*, 2503-2515.

Tsantoulas, C., Lainez, S., Wong, S., Mehta, I., Vilar, B., & NcNaughton, P. A. (2017). Hyperpolarization-activated cyclic nucleotide–gated 2 (HCN2) ion channels drive pain in mouse models of diabetic neuropathy. *Science Translational Medicine, Vol. 9*, 1-30.

Tu, H., Zhang, L., Tran, T. P., Muelleman, R. L., & Li, A. Y. (2010). Diabetes alters protein expression of Hyperpolarisation-activated Cyclic Nucleotide-Gated chhannel subunits in rat nodose ganglion cells. *Neuroscience, Volume 165*, 39-52.

Young, G. T., Emery, E. C., Mooney, E. R., Tsantoulas, C., & McNaughton, P. A. (2014). Inflammatory and neuropathic pain are rapidly suppressed by peripheral block of hyperpolarisation-activated cyclic nucleotide-gated ion channels. *Pain, Vol. 155*, 1708-1719.

Figure legends (organised chronologically)

Figure 1:

Figure 1 – Diagram of stimulating and recording scenarios for recording of upper and lower limb nerve excitability. Figure 1A shows recording for the upper limb median nerve, with the active and recording electrodes over the belly and distal point of APB respectively. The median nerve was stimulated through a cathode at the wrist over the course of the median nerve with the anode placed at the mid-forearm proximally and laterally. The temperature probe is seen next to the cathode, housed in a cardboard shell. Figure 1B showed recording

for the lower limb tibial nerve, with the active and recording electrodes over the belly and distal point of AH respectively. The tibial nerve was stimulated through a cathode at the ankle inferior to the medial malleolus over the course of the tibial nerve with the anode placed over the bony protrusion of the tibia. A cylindrical plastic tube was secured over the cathode to compress the electrode and minimise the volume between stimulating electrode and nerve for stimulation. The temperature probe is seen slightly proximally to the cathode housed in a cardboard shell. Figure 1C demonstrates the warming procedure for the upper limb. The arm was submerged into a thermostatically controlled water bath after encapsulating within a polythene plastic sleeve. The arm was suspended using a material sling inside of the water bath which allowed water turbulence and ensured the limb was at the mid point in the water, not next to the heating element which would have caused focal heating. Figure 1D shows the same heating procedure for the lower limb, with the addition of the foam edging to ensure patient comfort during this time in this position.

Figure 2:

Figure 2 - Strength-duration properties in normal temperature and during hyperthermia in the same limb. Data is plotted as the group mean (open triangles) ± SEM (error bars). A: Strength-duration properties of the median nerve at normal temperature (green) and during hyperthermia (magenta). B: Strength-duration properties of the tibial nerve at normal temperature (grey) and during hyperthermia (red).

Figure 3:

Figure 3 – RC curves during temperature change, data is expressed as group mean (circles) ± SEM (error bars). A: RC curve of the median nerve at normal temperature (green) and in hyperthermia (magenta). B: RC curve of the tibial nerve at normal temperature (grey) and in hyperthermia (red). C: RC curves during hyperthermia of the median nerve (magenta) and tibial nerve (red). D: Diagram of RC curve indices with (Relative Refractory Period), SNP (Super-normal period) and LSP (Late Sub-excitable Period).

Figure 4:

Figure 4 – Threshold electrotonus between temperature variables. Data is expressed as group mean (open circles) ± SEM (error bars). A: TE of the median nerve during normal (green) and hyperthermic (magenta) temperatures. B: TE of the tibial nerve during normal (grey) and hyperthermic temperatures (red). C: TE of median (magenta) and tibial (red) nerves during hyperthermia. The upper red arrow indicates TEd 40-60ms and the lower red arrow indicates S3 at TEh -100% which were found to demonstrate differential responses between the upper and lower limbs.

Figure 5:

Figure 5 - Current-voltage (I/V) relationship for normal and hyperthermic temperatures. Data is expressed as the mean ± SEM. A: I/V curve in the median nerve during normal temperature (green) and during hyperthermia (magenta). B: I/V curve in the tibial nerve during normal temperature (grey) and during hyperthermia (red). C: I/V curve in the hyperthermic median (magenta) and hyperthermic tibial (red) nerves.

Journal of Neurophysiology – Manuscript Figures V5.33

Date: 28th January, 2018

Manuscript title:

Upper and lower limb motor axons demonstrate differential excitability and accommodation to strong hyperpolarising currents during induced hyperthermia

Corresponding author:

Oliver R Marmoy

Comment:

The following figures are in reference to our manuscript submitted to the Journal of Neurophysiology (ref. JN-00464-2018). Five images are attached with figure legends detailed within the article file as per peer-review coordinator instructions.

Figure 1

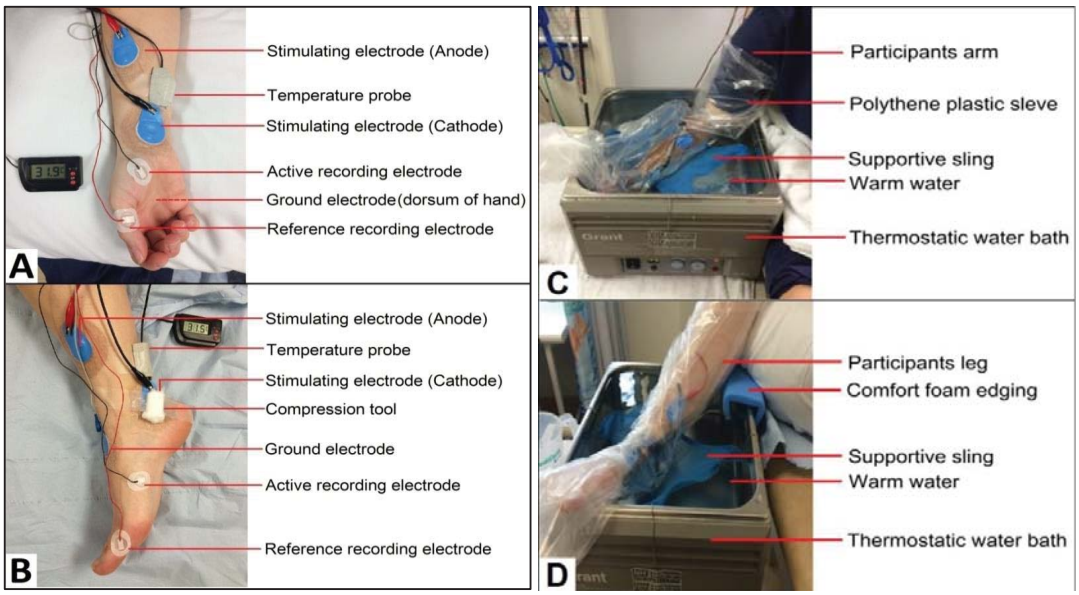

Figure 2

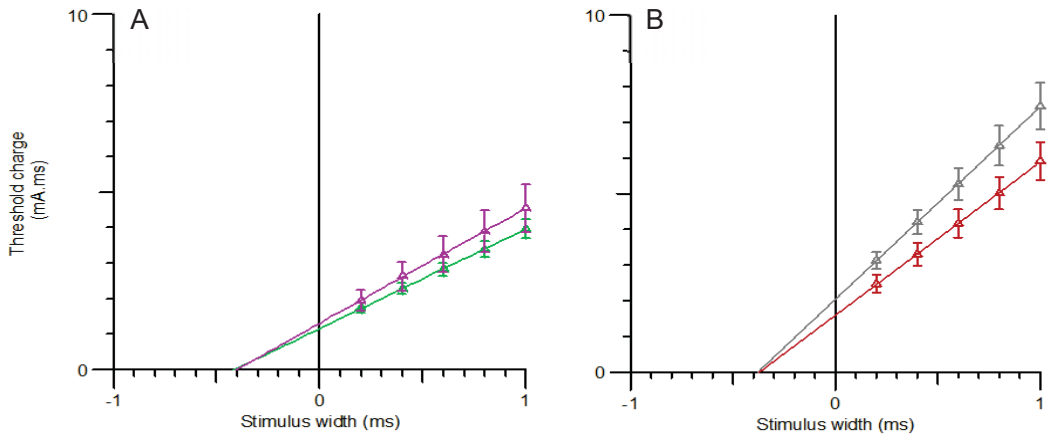

Figure 3

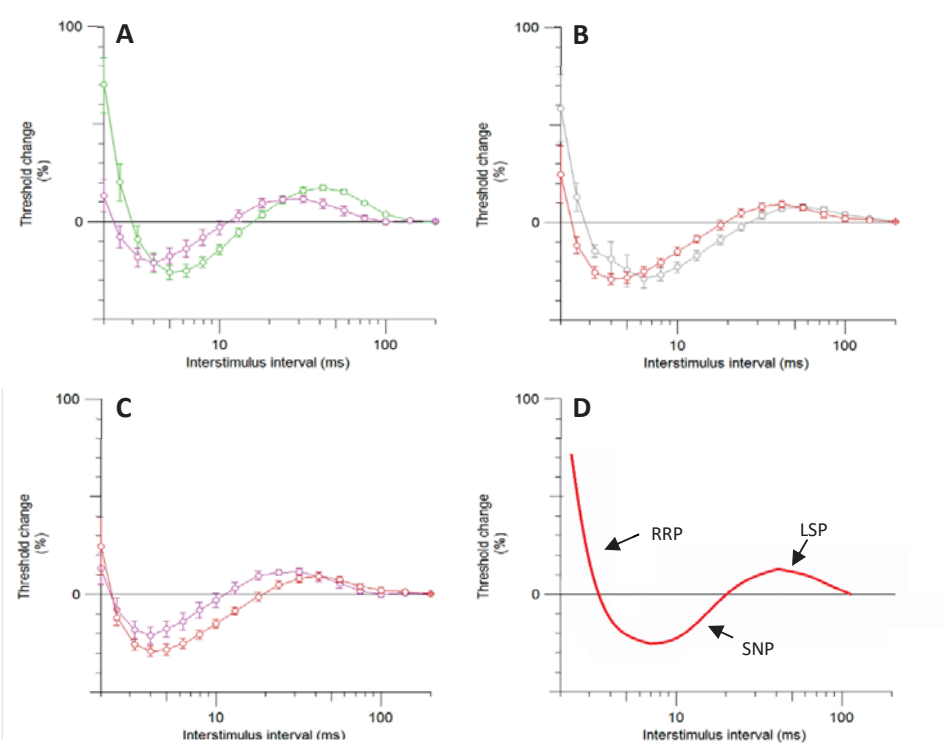

Figure 4

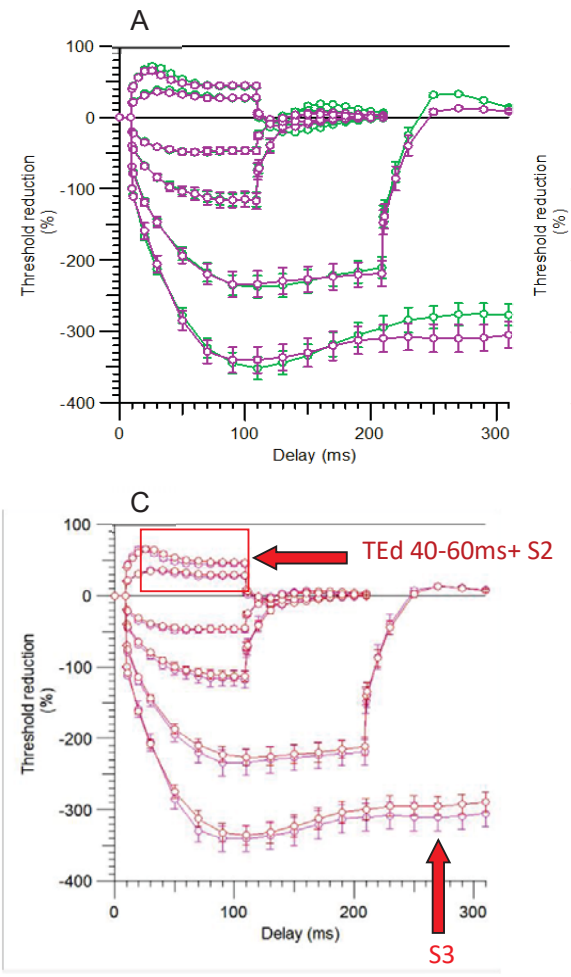

Figure 5