Mechanisms of axonal dysfunction in diabetes mellitus

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Abstract

This thesis explores the pathophysiology of axonal dysfunction in diabetes, utilizing excitability techniques which provide information on axonal ion channel function in human subjects. The rationale was that excitability studies may be useful to determine the mechanisms underlying axonal dysfunction in diabetic peripheral neuropathy (DPN) and that it may serve as a biomarker of incipient neuropathy and possibly as a means of monitoring treatment efficacy.

Excitability studies were initially undertaken in 54 patients with type 2 diabetes (T2DM), and demonstrated a relationship between neuropathy-specific-quality-of-life and excitability markers that reflect activity of persistent Na+ conductances. These changes occurred concurrently with progressive axonal depolarization with increasing neuropathy severity. Further studies were then undertaken to explore these mechanisms in type 1 diabetes (T1DM). Assessment of sensory and motor excitability in 30 patients suggested membrane depolarization in sensory and motor axons. Mathematical modelling demonstrated that these changes were due to reduced nodal Na+ and K+ conductances and abnormal Na+/K+pump activity.

Having demonstrated prominent changes in axonal function in T1DM, studies were conducted to explore the basis for these changes. The possibility that different forms of insulin administration may have differing effects on axonal function was considered. Axonal function was assessed in two separate cohorts of T1DM patients: those treated with continuous subcutaneous insulin infusion (CSII) and a second cohort who received multiple daily insulin injections (MDII). The studies demonstrated abnormalities of axonal function in MDII-treated patients. In contrast, CSII-treated patients had normal axonal function.

The final series of studies explored the effect of glycaemic variability on axonal function in T1DM. The relationship between glycaemic variability and axonal excitability was assessed in 12 T1DM patients, using a continuous glucose monitoring system. Patients were studied at three different glucose ranges and glycaemic variability was separately measured over a 48-hour period at the time of testing. The studies demonstrated that acute glucose level did not correlate with axonal dysfunction. However, glycaemic variability was strongly correlated with neurophysiological parameters, suggesting that it is an important determinant of axonal dysfunction in T1DM.

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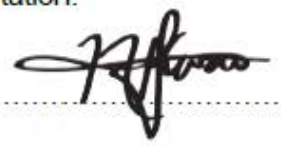
Signed 

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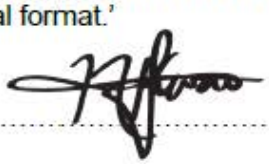
Signed 

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Abstract

This thesis explores the pathophysiology of axonal dysfunction in diabetes, utilizing excitability techniques which provide information on axonal ion channel function in human subjects. The rationale was that excitability studies may be useful to determine the mechanisms underlying axonal dysfunction in diabetic peripheral neuropathy (DPN) and that it may serve as a biomarker of incipient neuropathy and possibly as a means of monitoring treatment efficacy.

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Publications

Publications directly resulting from studies undertaken in this doctorate

Literature review:

Arnold R, Kwai NC, Krishnan AV. Mechanisms of axonal dysfunction in diabetic and uraemic neuropathies. Clin Neurophysiol. 2013;124(11):2079-90.

Chapter 1:

Kwai NC, Arnold R, Wickremaarachchi C, Lin CS, Poynten AM, Kiernan MC, Krishnan AV. Effects of axonal ion channel dysfunction on quality of life in type 2 diabetes. Diabetes Care. 2013;36(5):1272-7.

Chapter 2:

Kwai N, Arnold R, Poynten A, Howells JT, Lin CSY, Kiernan MC, Krishnan AV. In vivo evidence of reduced nodal and paranodal conductances in type 1 diabetes. Submitted to *Diabetes 23/01/2015.*

Chapter 3:

Kwai N, Arnold R, Poynten A, Lin CSY, Kiernan MC, Krishnan AV. Continuous subcutaneous insulin infusion (CSII) preserves axonal function in Type 1 diabetes mellitus. Diabetes Metab Res Rev. 2014.

Associated publications achieved during candidature

Arnold R, Kwai N, Lin CS, Poynten AM, Kiernan MC, Krishnan AV. Axonal dysfunction prior to neuropathy onset in type 1 diabetes. Diabetes Metab Res Rev. 2013;29(1):53-9.

Sung JY, Park SB, Liu YT, Kwai N, Arnold R, Krishnan AV, et al. Progressive axonal dysfunction precedes development of neuropathy in type 2 diabetes. Diabetes. 2012;61(6):1592-8.

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Awards

2011-2014 Australian Postgraduate Award

2013 Travel Award, Australian Diabetes Society

2013 Travel Award, School of Medical Sciences, Faculty of Medicine

2013 March Paper of the Month, School of Medical Sciences, Faculty of Medicine, UNSW

Presentations

2011 Australian Diabetes Society and Australian Diabetes Educators Association Annual Scientific Meeting (Perth, Australia, August 2011) - Junior Investigator Award session.

Neurophysiological parameters and impaired quality of life in diabetic peripheral neuropathy (DPN).

2011 14th Australian and New Zealand Association of Neurologists Clinical Neurophysiology Workshop (Gold Coast, Australia, October 2011). .

Effects of altered axonal biophysical properties and impaired Quality of Life in Diabetic Peripheral Neuropathy.

2012 13th Australian Oceanic Congress of Neurology (Melbourne, Australia, June 2012).

Altered sensory nerve excitability may underlie neuropathy development in type 1 diabetes.

2012 Australian Diabetes Society and Australian Diabetes Educators Association Annual Scientific Meeting (Gold Coast, Australia, October 2012) - Junior Investigator Award Session.

Neuroprotective potential of insulin pump therapy in type 1 diabetes mellitus.

2012 7th World Congress on Prevention of Diabetes and its Complications (Madrid, Spain, November 2012).

Neuroprotective potential of insulin pump therapy in type 1 diabetes mellitus.

2013 15th Australian New Zealand Association of Neurologists Clinical Neurophysiology Workshop (Gold Coast, Australia, October 2013).

Insulin pump therapy in type 1 diabetes: potential to be neuroprotective.

2014 42nd Annual Coast Association Tow Research Awards Day Senior Investigators' session (Sydney, Australia, November 2014).

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Abbreviations

BGL	blood glucose level
C-peptide	connecting peptide
CSII	continuous subcutaneous insulin infusion
CGM	continuous glucose monitoring
CNS	central nervous system
DPN	diabetic peripheral neuropathy
DCCT	Diabetes Control and Complications Trial
EDIC	Epidemiology of Diabetes Interventions and Complications study
HbA1c%	percentage glycosylated haemoglobin
K^+	potassium
MDII	multiple daily insulin injections
Na^+	sodium
Na^+/K^+pump	sodium potassium pump/sodium potassium ATPase
Na_p	persistent sodium conductance
NeuroQoL	Neuropathy-specific Quality of Life Questionnaire
NCS	nerve conduction study
PNS	peripheral nervous system
SR	stimulus-response
TE	threshold electrotonus
TEd	depolarizing threshold electrotonus
TEh	hyperpolarizing threshold electrotonus
TNS	Total Neuropathy Score
TNG	Total Neuropathy Grade
τ_{SD}/SDTC	strength-duration time constant

Literature Review

THE HUMAN NERVOUS SYSTEM

The nervous system is anatomically divided into two components; the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS defines neuronal tissue that resides in the brain and spinal cord whilst the PNS encapsulates tissue residing outside of these regions including spinal nerves. There are two primary classes of cells that form the physical components of the nervous system. The first is the neuron which is the conduit of impulse propagation and conduction and glial cells which primarily provide neurotrophic support.

The neuron

A neuron is a specialized unit that allows for the propagation and conduction of electrical impulses termed *action potentials*. The metabolic centre and cell body of the neuron is termed the *soma* which houses the cell nucleus, rough endoplasmic reticulum and Golgi bodies that provide the means for maintenance of the axon (Kandel, 1991, Berthold *et al.*, 2005). Short branch like extensions of the soma cytoplasm are termed *dendrites,* and provide the principle structures for reception of incoming signals from other neurons. The relaying conduit of the neuron is termed the *axon* and is a long projection to either another neuron or effector organ from the soma. The axon accounts for at least 95% of the neuron's cell mass (Miller, 2014), and importantly, is involved in impulse *propagation* and *conduction.*

THE PERIPHERAL AXON

The primary function of the peripheral axon is to rapidly propagate and transmit electrical signals to an effector organ or other neurons. As a secondary function, the axon provides a pathway for biological materials such as proteins and neurotransmitters to support structure and function of the axon itself (Wang and He, 2013). These functions are made possible by the intuitive structural design and compartmentalization of the axon (Kandel, 2000, Poliak and Peles, 2003), which, although only 1 – 2μm wide in the case of large myelinated fibres, are able to maintain axonal *membrane potential* and generate electrical impulses termed *action potentials.* Specifically, impulse conduction is made possible through the organization of insulating sheathing called *myelin*, and the presence of *voltage-gated ion channels, ionic pumps* and *exchangers* (Berthold *et al.*, 2005).

Structure of the myelinated peripheral axon

The initial segment of the *axon* arises from the soma of the neuron at the *axon hillock*, which demarcates the boundary between the somatodendritic domain and the axonal domain of the neuron (Jones *et al.*, 2014) (Figure 1a). This is also the primary site of action potential generation (Kandel, 1991). The neuronal cytoplasm or *axoplasm* is contained by the axonal cell membrane, the *axolemma.* The axolemma is ~6-8nm thick and comprised of a phospholipid bilayer with a hydrophobic core and hydrophilic internal and external walls (Siegelbaum and Koester, 1991). Positively charged ions are electrostatically attracted to oxygen in water molecules of the extra-axonal fluid. These molecules must thus pass through highly selective *ion channels* as the axolemma is impermeable to charged ions. This is due to the uncharged hydrocarbon tails of lipid bilayer, which do not provide sufficient energy to attract

charged ions or separate them from the water molecules they are bound to in solution (Siegelbaum and Koester, 1991) (Figure 1c).

In myelinated axons, the axolemma is periodically ensheathed by spiral lamellae of *Schwann cell* cytoplasm (Figure 1b). Schwann cells are the peripheral neuroglia comparable to the *oligodendrocyte* of the central nervous system and maintain a proximity of no more than 30nm to the axolemma in ensheathed areas (Berthold *et al.*, 2005). Schwann cells produce myelin which provides an insulating barrier that prevents current leakage from the *internodal* regions of the axon and which increases the resistance of the axonal membrane (Nave and Werner, 2014).

The myelin sheath also has a crucial role in the separation and demarcating of primary structural and functional domains of the axon (Figure 1d). The junction of neighbouring Schwann cells on the axolemma remains unmyelinated and is referred to as the *node of Ranvier*. Covered by microvilli of the outermost layer of the Schwann cell, the node of Ranvier spans ~1μm and is highly populated by voltage-gated sodium (Na^+) channels (1500μm^2) (Poliak and Peles, 2003, Berthold *et al.*, 2005). This ion channel distribution is similar to the axon hillock (Rasband, 2010) and consequently, the node of Ranvier is the site for regeneration of the action potential in saltatory conduction (Hille, 2001, Susuki, 2013). The node is flanked by the *paranodal region*, a 3-5nm region of the axon where Schwann cell attachment to the axolemma occurs and myelination begins/terminates (Berthold *et al.*, 2005). Within this region, contactin and contactin-associated protein (Caspr) form *axo-glial junctions* between Schwann cells and the axolemma. Pathologically, axo-glial dysfunction or disorganization results in spatial redistribution of ion channels between the node and internodal regions (Rosenbluth, 2009), with subsequent

impairment of impulse conduction (Dupree *et al.*, 1999, Rasband *et al.*, 1999, Susuki *et al.*, 2007, Salzer *et al.*, 2008, Babbs and Shi, 2013). Neighbouring the *paranode* is the *juxtaparanode*, an area that is ensheathed by compact layers of myelin and which houses Shaker-type K^+ channels (Kv1.1 and 1.2) that are involved in repolarization of the action potential (Salzer *et al.*, 2008). The remaining 95% of the *internodal* region, or simply the *internode*, is the largest domain of the peripheral axon with lengths from 0.15 – 1.2 mm (Lascelles and Thomas, 1966, Salzer *et al.*, 2008). The internode is tightly sheltered by compact myelin sheathing and contains a number of axonal ion channels, pumps and exchangers involved in maintenance of membrane potential and impulse conduction (Krishnan *et al.*, 2009).

Function of peripheral myelinated axons

The elaborate architecture of the myelinated axon permits the propagation and *saltatory conduction* of *action potentials*. This electrical phenomenon defines a discrete series of changes in voltage of the axon produced by Na^+ and K^+ conductances across the cell membrane (Hodgkin and Huxley, 1952). Classical studies by Hodgkin, Huxley and Katz on the giant squid axon provided the physiological foundation for an enduring mathematical model of axonal conduction. Their model proposed that saltatory conduction was dependent on the permeability of the axolemma to Na^+ and K^+ ions (Hodgkin and Katz, 1949, Hodgkin and Huxley, 1952, Hodgkin and Huxley, 1952).

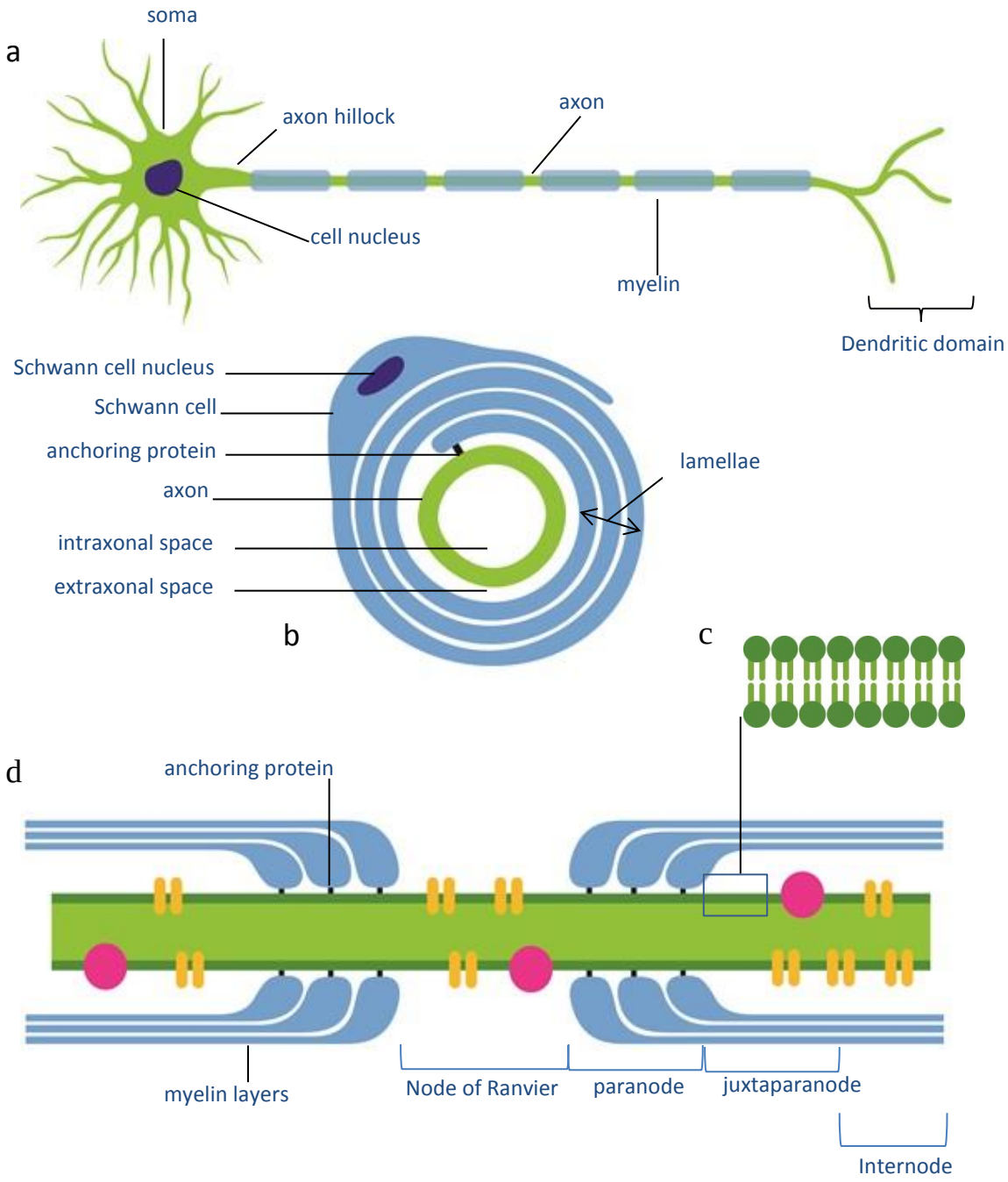

Figure 1. Schematic of a neuron, Schwann cell, phospholipid bilayer and regions of a myelinated axon

A neuron (a) has a soma which houses the nucleus, a long projection of its cytoplasm termed the axon and branch like projections called dendrites. The axon is wrapped periodically by multilamellar layers of Schwann cell cytoplasm (b) which forms the myelin sheath, providing insulation for impulse conduction. The axon membrane or axolemma is formed by a phospholipid bilayer with a hydrophobic core (c). The regions of the axon can

be demarcated into functionally distinct regions: node of Ranvier (site of impulse propagation), paranode (are of myelin attachment), the juxtaparanode and the internode. Pink circles schematically represent pumps and exchangers on the axolemma (further detail is provided in Figure 3).

Membrane potential

Axons possess a relatively negative charge intra-axonally compared to the extra axonal space (Koester, 1991). The difference is designated *membrane potential* and is a fundamental determinant of action potential propagation (Hodgkin and Huxley, 1952). Conventionally, membrane potential can be represented as an equation:

$$V_m = \textit{voltage inside the axon } (V_{in}) - \textit{voltage outside the axon } (V_{out})$$

At 'rest' the axonal membrane potential is ~-60mV to – 70mV due to higher concentrations of Na^+ and Cl^- ions extra-axonally and higher concentrations of K^+ and organic anions intra-axonally (Koester, 1991). The charge separation is carefully maintained by the selective permeability of the axolemma's bilipid structure, the voltage dependence of ion channels and the activity of energy dependent pumps which re-establish ionic gradients following an action potential (Koester, 1991). Experimental models in regenerating axons indicate changes in membrane potential that promote slower recovery of impulse conduction (Moldovan and Krarup, 2004). Alteration of the membrane potential causes marked changes in an axon's ability to propagate and conduct an action potential (Hodgkin and Katz, 1949, Hodgkin and Huxley, 1952) and has been implicated in a number of neurological disorders including forms of peripheral neuropathy (Kiernan *et al.*, 2002a, b, Krishnan *et al.*, 2006, Arnold *et al.*, 2013).

The Action Potential

The action potential is a regenerative impulse that is defined by a series of discrete changes in voltage across the axolemma. Broadly, the events defining its time course can be divided into three stages of change in membrane potential (Figure 2).

(i) *Depolarization.* In response to a chemical or electrical stimulus, a small number of voltage-gated Na^+ channels in the axon hillock or node of Ranvier open, allowing an inward current of Na^+ from the extra-axonal space (Hodgkin and Katz, 1949, Koester, 1991, Koester, 1991, Barnett and Larkman, 2007). This produces an increase in membrane potential or *depolarization* from rest (~ - 60mV) until a critical level of voltage is reached, termed *threshold* (~ -45mV). Once threshold is achieved, the remaining pool of voltage-gated Na^+ channels open allowing Na^+ to flood rapidly into the axon and accounting for the rapid upstroke in membrane potential which typically lasts ~1ms. This *all or none* response contributes to the *regenerative* nature of the action potential itself (Barnett and Larkman, 2007).

(ii) *Hyperpolarization.* During this phase, the membrane potential returns slowly back to resting levels. This is achieved by closure of voltage-gated Na^+ channels and the opening of voltage-gated K^+ channels. Additional efflux of K^+ through these channels coupled with the significant inactivation of Na^+ channels, induces a rectifying hyperpolarization of the axonal membrane (Koester, 1991, Koester, 1991).

(iii) The final phase of the action potential is characterized by the return of the membrane potential to resting values. This first component of this phase encapsulates a *hyperpolarizing overshoot* of the membrane potential, due to the slow inactivation of the voltage-gated K^+ channels and subsequent K^+ leak. This causes the membrane

potential to drop below resting levels. Return to resting state occurs when all voltage-gated ion channels are closed and membrane potential is restored via the activity of the Na^+/K^+ pump.

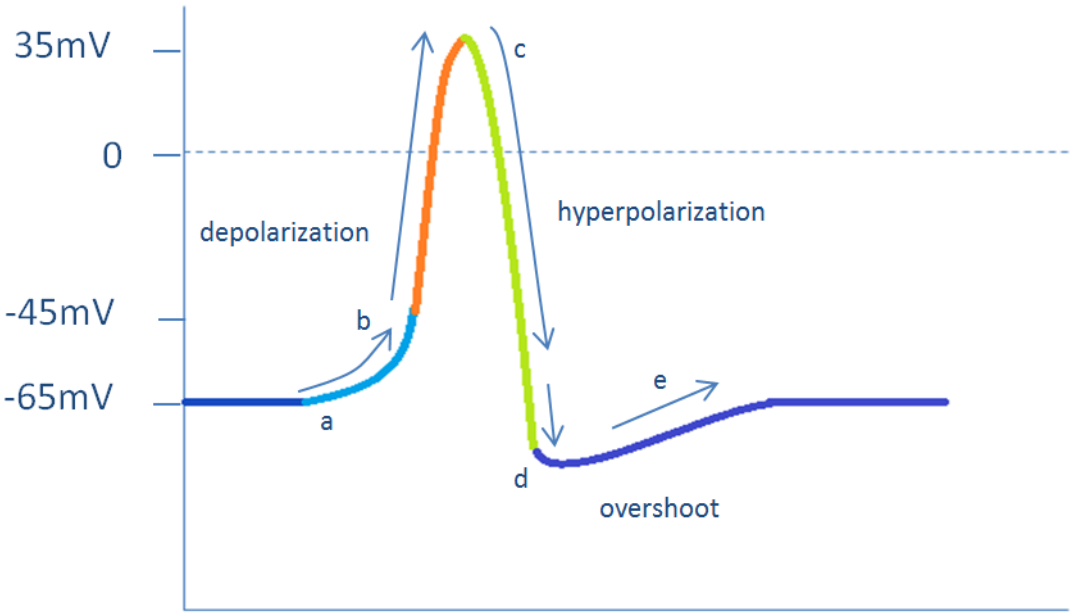

Figure 2. Action potential: sequence of events

Following a stimuli depolarization occurs whereby (a) membrane voltage increases to threshold after which (b) rapid upstroke of membrane potential occurs due to the activation of voltage-gated Na^+ channels. Following this, hyperpolarization occurs (c) as a result of inactivation of Na^+ channels, arresting membrane voltage increase and activation of K^+ channels. *Overshoot* of resting membrane voltage occurs (d) due to slow inactivation of K^+ channels and returns to rest as voltage-gated ion channels return to their resting state and is maintained by the Na^+/K^+ pump (e).

Saltatory conduction

Swift conduction of an action potential generated at the *axonal initial segment* (Rasband, 2010) and without attenuation along the axon is termed *saltatory conduction*. First described by Lillie (1925), current passing through the axon travels through the internode and initiates an action potential at the next node of Ranvier (Lillie, 1925, Huxley and Stämpeli, 1949). This is facilitated by high resistance of the internodal membrane achieved by compact myelin sheathing and maintenance of a high density of Na^+ channels at the node of Ranvier (Rosenbluth, 2009, Young *et al.*, 2013). Impulse generation at the node or Ranvier is >5 times that needed to generate an action potential, safeguarding against any leakage of current or any capacitance of the internode until the current reaches the succeeding node (Tasaki, 1939, Huxley and Stämpeli, 1949, Stampfli, 1954). This is termed the *safety factor of transmission* and can be calculated as a ratio of current leaving the node of Ranvier to current required for excitation at the next node (Hodgkin, 1937, Tasaki, 1939). A reduction in safety factor may occur due to demyelination and can induce conduction block and the development of neurological symptoms such as weakness (Kuwabara and Yuki, 2013).

Ionic conductances: channels, pumps and exchangers

The biophysical foundations for effective impulse conduction in the peripheral nervous system is the presence of a number of voltage-gated ion channels, energy dependent ion pumps and exchangers on the axolemma (Siegelbaum and Koester, 1991). These protein complexes permit ions to either flow down their concentration gradient, potentiating an action potential, or pump ions against their concentration gradient for maintaining axonal membrane potential (Tien *et al.*, 2014)(Figure 3).

Ion channels

Efficient and rapid alterations in membrane potential during action potential genesis is mediated by a class of integral membrane proteins called *ion channels* (Siegelbaum and Koester, 1991). Ion channels are protein complexes that span the axolemma and contain a pore to allow selective permeation of ions across the axolemma, thus contributing to impulse generation (Catterall, 1993, Tien *et al.*, 2014). Three basic states of ion channel activity make this possible: open, closed and inactivated. The shifting between states is termed *gating* which is 'biphasic' – either open or closed (Catterall, 1993). Of the various forms of ion channels present in the human nervous system, voltage-gated channels are most relevant to this thesis. These undergo conformational change in response to change in voltage - either depolarization or hyperpolarization of the membrane potential of the axon (Catterall, 1988, Catterall, 1993).

Voltage-gated Na^+ channels

Landmark studies used covalent labelling of neurotoxin receptor sites to determine the molecular structure of axonal voltage-gated Na^+ channels (*Na_v)* (Beneski and Catterall, 1980). These and more recent studies revealed that these channels have a number of reactive residues: an α subunit (~260kDa) of four homologous domains (I-IV) and one or two singular smaller β subunits (~30-40kDa) (Beneski and Catterall, 1980, Catterall, 1988, Hille, 2001, Catterall, 2012)(Figure 3). Each domain has six membrane spanning segments (S1-S6) with a re-entrant pore loop between S5 and S6 domains, which forms the pore of the channel (Catterall *et al.*, 2007) Voltage activation of the Na_v is via the S4 segment. This sensor region is comprised of repeated charged amino acid residues and hydrophobic residues that under the presence of depolarization, undergo mechanical extrusion, causing a conformational change of the ion channel and subsequently opening the pore (Catterall *et al.*, 2007).

Functional, pharmacological and structural characterization of Na_v have revealed 9 isoforms defined by their α subunit (Goldin *et al.*, 2000). Nomenclature is based on these variations such that channels are named by their α subunit isoform, denoted $Na_v1.1$ – $Na_v1.9$ (Goldin *et al.*, 2000). Of greatest density in the peripheral nervous system are $Na_v1.7$, $Na_v1.8$ and Na_v 1.9 (Goldin, 2001). Of importance, the channel isoform $Na_v1.6$, found in the central nervous system is also expressed in the peripheral nervous system (Beckh, 1990, Schaller *et al.*, 1995, Dietrich *et al.*, 1998) and is the dominant isoform at the node of Ranvier (Caldwell *et al.*, 2000). This channel isoform is therefore a key contributor to action potential propagation.

Two functionally distinct types of Na^+ current have been identified; a transient Na^+ current that activates in response to depolarization and inactivates to inhibit further flow of current and thus further depolarization (Burke *et al.*, 2001). Approximately 98% of Na^+ channels exhibit this functional characteristic. The remaining 2% activate at ~10-20mV below the activation threshold of the transient conductances and are termed "persistent" Na^+ conductances (Na_p). These conductances undergo incomplete inactivation and contribute an inward leakage of Na^+ ions at resting membrane potential. Na_p is thus linked to spontaneous neuronal firing (Taddese and Bean, 2002), and is implicated in ectopic symptom generation (Mogyoros *et al.*, 1997). Consequently, a large class of neurological medications targeting modulation of Na_v are used as a means of treating ectopic symptomatology (Eijkelkamp *et al.*, 2012)

Voltage-gated K^+ channels

Flow of K^+ ions outward terminates excitation during the action potential and attenuates further depolarization. Voltage-gated K^+ channels (K_v) form the largest family of K^+ selective channels amongst calcium (Ca^{2+}) activated (K_{Ca}), inward rectifying (K_{IR}) and two-pore (K_{2P}) K^+ channels (Gutman *et al.*, 2005). Twelve distinctive subfamilies exist, K_v1 – K_v12, named in accordance with their amino acid sequence homology (Gutman *et al.*, 2005). Typically, the K_v is conical in shape, is homo- or heterotetrameric and comprised of six transmembrane helices (S1 - S6) (Yellen, 2002, Catterall *et al.*, 2007). It has N and C termini on the intraxonal portion of its structure and a narrow selectivity filter (3- 3.3Å wide) (Hille, 1973, Yellen, 2002). The voltage sensor is located at S4 and with depolarization, conformational change results in the extrusion of this segment into the extracellular

space thus opening the channel (Larsson *et al.*, 1996). Selectivity of these channels for K^+ ions is attributed to the presence of stabilizing structures present in the channel that are specific to K^+ ions: water molecules in the initial dilated region of the channel, helix dipoles that create electrostatic force to push ions towards the narrow region of the filter and carbonyl oxygen atoms of the selectivity loop that mimic the water cage naturally assumed by K^+ ions in solution (Yellen, 2002). High conduction rates for the K_v is thought to be due to high electrostatic repulsion between K^+ ions that rapidly pass in a single file through the pore (Hille, 1973).

Two functionally distinct types of K_v are relevant to this thesis; those possessing fast kinetics and slow kinetics. *Fast K_v* are involved primarily in limiting the extent of depolarization during the action potential (Barrett and Barrett, 1982). They are active at higher levels of membrane depolarization (-40 - +40mV) and have a density six times higher in the juxtaparanodal region of the axon compared to the node and internode (Roper and Schwarz, 1989, Wang *et al.*, 1993) (Figure 3). The primary isoform of the fast K_v in the peripheral nerve is K_v1.1, which is encoded by the KCNQ1gene. Mutations in this gene have been implicated in diseases of neuronal hyperexcitability such as epilepsy and episodic ataxia (Devaux *et al.*, 2004, Tomlinson *et al.*, 2010). *Slow K_v* are primarily expressed in the nodal region (Devaux *et al.*, 2004) and are activated at potentials as negative as -110mV (Roper and Schwarz, 1989, Schwarz *et al.*, 2006). A proportion of these channels are thus active at rest (~35%) (Roper and Schwarz, 1989) and subsequently play an important role in maintenance of the resting membrane potential (Krishnan *et al.*, 2009). Furthermore, their activity produces the hyperpolarizing afterpotential that forms the late subnormal period of impulse recovery in axons (Baker *et al.*, 1987). Slow K_v has

been ascribed to KCNQ2 and KCNQ3 genes (Wang *et al.*, 1998, Schwarz *et al.*, 2006) which encode for $K_v7.2$ and $K_v7.3$(Gutman *et al.*, 2005). As with KCNQ1, mutations of KCNQ2 and KCNQ3 are associated with epilepsy phenotypes in adults and neonates (Claes *et al.*, 2004, Weckhuysen *et al.*, 2012, Soldovieri *et al.*, 2014).

Inward rectifiers

A class of ionic conductances termed *inward rectifiers ($I_{h,}$)*, have also been described in axons (Figure 3) . Their primary function is to limit the extent of hyperpolarization (Pape, 1996). This "queer" property is unlike archetypal voltage-gated ion channels which typically activate in response to depolarization, (Araki *et al.*, 1962, Moosmang *et al.*, 2001). Functionally, I_h describes an inward flow of only K^+ or a combination of K^+ and Na^+ (Baker *et al.*, 1987, Moosmang *et al.*, 2001), occurring at 30ms after depolarization and generally reaching a steady state between 100 and 200ms (Waxman *et al.*, 1995). Maximal activation of these conductances occur at membrane potentials of ~ -110mV (Pape, 1996) .

The channel of most relevance to the peripheral nervous system is the hyperpolarization activated cyclic-nucleotide cation (HCN) channel. HCN is termed a "pacemaker" channel and lies within the K^+ superfamily (Hibino et al., 2010). Similar to Shaker type K^+ channels (Hibino *et al.*, 2010), they are composed of six transmembrane spanning α helices, with a cyclic- nucleotide binding domain in the C-terminus and a voltage sensor at the S4 domain (Jiang *et al.*, 2008). Cyclic AMP (cAMP) or cyclic GMP (cGMP) binds to the cyclic-nucleotide binding domain at the C-terminus of the channel to activate it.

There are four cloned isoforms of the HCN(1-4) (Ludwig *et al.*, 1998, Hogan and Poroli, 2008), however, HCN1 and HCN2 are the most densely expressed in neurons (Chaplan *et al.*, 2003, Momin *et al.*, 2008, Momin and Wood, 2008, Acosta *et al.*, 2012). In situ hybridization studies by Grafe et al. (1997) localized these channels to the internodal region of the membrane (Grafe *et al.*, 1997). Variations in the modulation and expression of HCN channels in sensory compared to motor axons have provided some explanation for their functional differences (Howells *et al.*, 2012).

Mutation of HCN channels resulting in upregulation has been linked to repetitive neuronal firing and neuropathic pain syndromes and are thus an avenue of exploration for novel drug therapies in pain management (Brown *et al.*, 2004, Jiang *et al.*, 2008, Postea and Biel, 2011).

Potassium selective inward rectifying channels (K_{ir}) are another class of I_h. Similar to HCN, activation of these channels is triggered by hyperpolarization of the axonal membrane potential, allowing a membrane depolarizing K^+ current and triggering Na_p currents (Leao *et al.*, 2012). There are seven subfamilies of K_{ir} which are subdivided further into four functional groups according to gating; G protein-gated K_{ir} channels (GIRK), ATP sensitive (K_{ATP}) and, classical/transport channels (Hibino *et al.*, 2010). As per HCN channels, functional mutations of the channels' isoforms has been linked to epilepsy and pain phenotypes (Ma *et al.*, 2010, Leao *et al.*, 2012, Li *et al.*, 2013).

Sodium potassium pump

Maintenance of axonal membrane potential is dependent on a number of factors as discussed above. However, a crucial determinant of the electrochemical gradient in the peripheral nervous system is the electrogenic *sodium potassium pump (Na^+/K^+ pump or Na^+/K^+ATPase*). The membrane bound Na^+/K^+ pump utilizes energy cleaved from the hydrolysis of ATP to exchange three intracellular Na^+ for two extracellular K^+ ions against their concentration gradients (Figure 3). This produces a net outward current and a more negative potential intraxonally (Saez *et al.*, 2009, Gulledge *et al.*, 2013).

The structure of the Na^+/K^+ pump is mostly preserved amongst other P-type ATPase pumps, whereby it possesses a single α-subunit which undergoes phosphorylation and conformational transition, a β-subunit which anchors the α-subunit to the membrane, and a γ-subunit that provides regulation of pumping activity (Garty and Karlish, 2006). The pump is ubiquitous across different tissues, however, expression of different isoforms of the subunits occurs in a tissue-exclusive manner whereby α1, α3 and β1 are most populous in the CNS (Mata *et al.*, 1991, Watts *et al.*, 1991).

The requirements for ATP to promote adequate Na^+/K^+ pump function has been highlighted in animal studies showing >90% reliance on the presence of ATP (Erecinska and Dagani, 1990). Mutations of the Na^+/K^+ pump have been implicated in nerve conduction block and linked to neuromuscular pathologies such as transient hemiplegia, hemiparesis and dystonia (Heinzen *et al.*, 2012, Rosewich *et al.*, 2012, Kirshenbaum *et al.*, 2013)

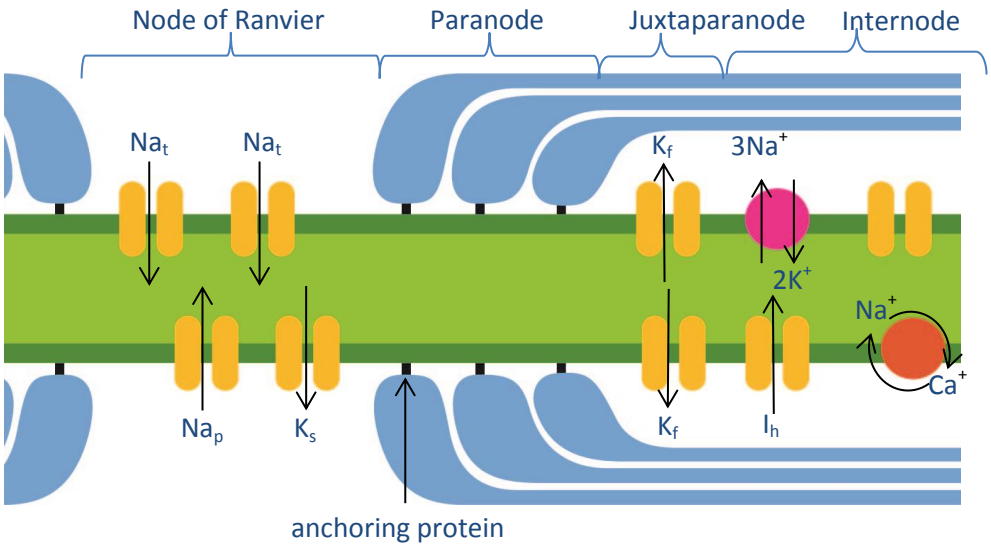

Figure 3. Schematic of a myelinated axon with ion channels, pumps and exchangers

There is a high density of Na^+ channels at the node of Ranvier; transient (Na_t) and persistent (Na_p). Slow K^+(K_s) is also expressed at the node. The paranodal region defines the junction between the axon and myelin (axo-glial junction). In the juxtaparanode, there is a high density of fast K^+ channels (K_f). Inward rectifiers (I_h) limit the extent of hyperpolarization. The Na^+/K^+ pump exchanges $3Na^+$ for $2K^+$ (pink circle) and the Na^+/Ca^+ exchanger regulates intra-axonal Ca^+ and is reversed in the presence of membrane depolarization which can lead to axonal death.(Lehning *et al.*, 1996).

Assessment of axonal function in the clinical setting

At present, the gold standard of objective measures for assessing peripheral axonal function in the clinical setting is nerve conduction studies (NCS). These studies provide a complement to clinical neurological examination in diagnosing peripheral nerve pathology. Methodologically, NCS involve maximal surface stimulation of a peripheral nerve, and recording the peak response from the innervated muscle belly or afferent by placing an active recording surface electrode on the skin over the site (Kimura, 1983).

The recordings obtained from NCS provide information on myelination and axon number such that a decreased conduction velocity indicates demyelination, and reduction in amplitude response broadly denotes loss of axons (Kimura, 1983, Kimura, 1984). NCS therefore provide robust diagnostic criteria for demyelinating and axonal neuropathies.

Despite their diagnostic utility, NCS are unable to provide further formative information on underlying causes of altered conduction in the peripheral nerve (Burke *et al.*, 2001, Quattrini *et al.*, 2007). Such include changes in axonal membrane potential and ion channel dysfunction (Kiernan *et al.*, 2005), important determinants of axonal function.

The use of threshold tracking techniques has been considered as a potential adjunct to the use of standard NCS. In the 1970's Joseph Bergmans proposed *threshold* measurements in human nerves (Bergmans, 1970). Bergmans discovered that surface stimulation could excite a single motor unit and that information regarding the biophysical properties of an axon's membrane potential may be derived. As such,

threshold was determined as the minimum voltage required to excite a nerve in at least three out of five stimulus applications.

Technical difficulties however hampered development of these methods and the need to manually adjust current in particular proved to be a major hurdle for routine use of these techniques in a clinical setting.

Axonal excitability assessment

The development of a computer assisted protocol, QTRAC, has allowed for automated threshold tracking to occur in the clinical setting (Digitimer, UK). The protocol automatically adjusts stimulus intensity to attain a target response that is set at ~ 40% of the maximal obtainable response. The stimulus required to reach this target is termed *threshold*. Utilizing several conditioning stimuli, responses are tracked as % changes in threshold. These are performed rapidly and allow the fast acquisition of information regarding the excitability of the peripheral axon *in vivo*.

The basic succession of the testing protocol involves measurement of stimulus response behaviour followed by four distinct testing paradigms utilizing various conditioning stimuli (Kiernan *et al.*, 2005): 1) strength-duration assessment, 2) threshold electrotonus, 3) current-threshold relationship and 4) the recovery cycle.

Stimulus response behaviour (SR) curves are generated in a dose response pattern by which the stimulus output is increased until the maximal compound muscle (motor excitability protocol) or sensory action potential (sensory excitability protocol) is achieved (Figure 4). The stimulus required to reach ~40% of the maximal response, or *threshold* is then calculated and remains the target for all succeeding test paradigms. The maximal stimulus response is comparable to NCS, and like standalone measures of threshold, provide similarly unremarkable pathological insights (Bostock *et al.*, 1998). However, the change in threshold (expressed as percentage threshold change for normalization purposes), after conditioning stimuli in the remaining paradigms provide important insights into the behaviour of voltage-gated conductances.

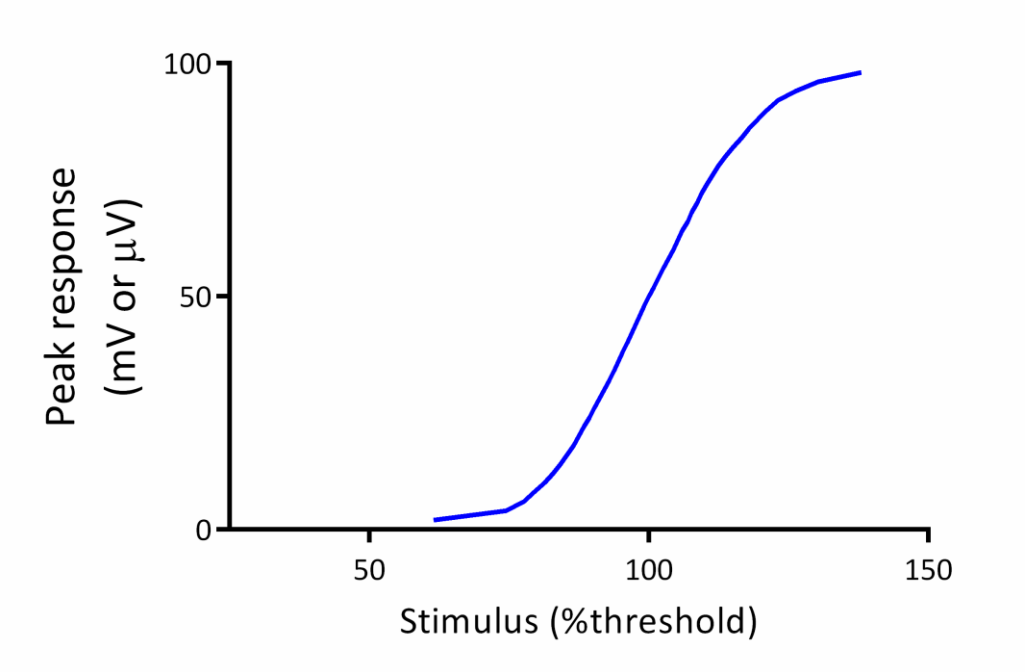

Figure 4. Stimulus response curve

The stimulus response curve plots the stimulus increase and corresponding increases in compound action potential (similar to a dose response curve). From this relationship, we can determine *threshold* or the stimulus required to reach a target response (~40% maximal response and defined as the stimulus that occurs at the steepest point on the curve). The stimulus response curve can be shifted to the left or right in the event of hyperpolarization or depolarization (respectively) of the axonal membrane potential.

Strength-duration properties (Figure 5) dictate that for increases in stimulus duration, intensity to elicit a target response decreases (Weiss, 1901) and this relationship is best defined by Weiss' Law (Mogyoros *et al.*, 1996, Bostock *et al.*, 2007). Weiss defined the relationship as a simple linear function: $Q = a + bt$ for which its simplicity was held in great regard (Lapicque, 1907). *Chronaxie*, an electrical property embedded within the strength-duration relationship, is defined as double *rheobasic current.* Chronaxie is interchangeable with *strength-duration time constant (SDTC)*, a marker which is sensitive to membrane potential (Bostock, 1983, Mogyoros *et al.*, 1996) and is a surrogate marker of Na_p function and nodal passive properties (Mogyoros *et al.*, 1996, Bostock and Rothwell, 1997).

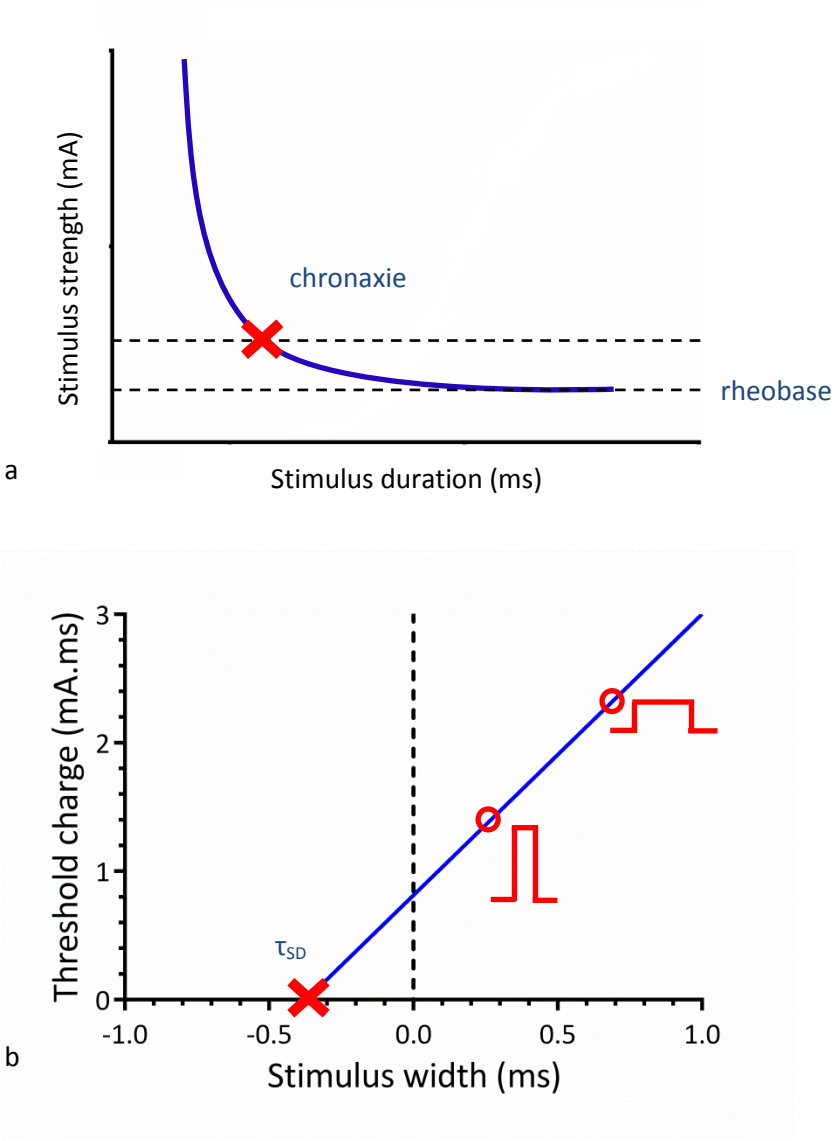

Figure 5. Strength-duration relationship

The hyperbolic relationship between stimulus intensity and stimulus duration is depicted in (a). Rheobase is defined as the stimulus intensity necessary for a stimulus of infinite duration whilst chronaxie is double rheobasic current. The linear relationship between stimulus duration and threshold is depicted in (b) as per Weiss' law, SDTC (red cross) is defined as the x-intercept of this relationship (analogous to chronaxie) and rheobase is interchangeable with the gradient of this relationship. *This figure was adapted from (Krishnan et al., 2009).*

Threshold electrotonus describes the electrical properties through the internodal region of the axon (Figure 6). The use of subthreshold conditioning pulses alters axonal membrane potential via capacitive charging of the internodal region (Baker *et al.*, 1987, Bostock *et al.*, 1998). Testing occurs via comparing threshold readings at rest, during and after the application of a subthreshold conditioning stimulus of 100ms at ± 40% threshold current (Burke *et al.*, 2001). Depolarizing subthreshold pulses initially cause a response that is proportional to the current applied, termed the *fast phase* or *F phase* (Bostock *et al.*, 1998, Krishnan *et al.*, 2009). This is limited by Na^+ conductances at the node of Ranvier. Following the fast phase is a slower change in threshold in the same direction termed S1 and is due to the spread of current into the internode and limited by juxtaparanodal fast K^+ currents (Krishnan *et al.*, 2008). Subsequently, the occurrence of an S2 phase draws excitability back towards control and is mediated by the slow closure of slow K_v of the internodal region. After the 100ms conditioning pulse, excitability drops towards and past control levels, the result of slow inactivation of rectifying K^+ conductances**.**

The hyperpolarizing phase of threshold electrotonus roughly follows the same pattern as the depolarizing phases but in the opposite direction. There is similarly an F phase in the hyperpolarizing direction in response to subthreshold hyperpolarizing conditioning pulses. However, the S1 phase is more pronounced as the slow K^+ conductances at the node are further activated by hyperpolarizing currents and with closure of fast K_v in response to hyperpolarization (Bostock and Rothwell, 1997, Bostock *et al.*, 1998). Thus the extent of hyperpolarization is not absolute until the activation of I_h.(S3 phase) (Pape, 1996, Bostock *et al.*, 1998). Finally, threshold

changes return and surpass the control threshold at tests after the 100ms pulse due to inactivation of I_h and activation of K^+ conductances (Krishnan *et al.*, 2009).

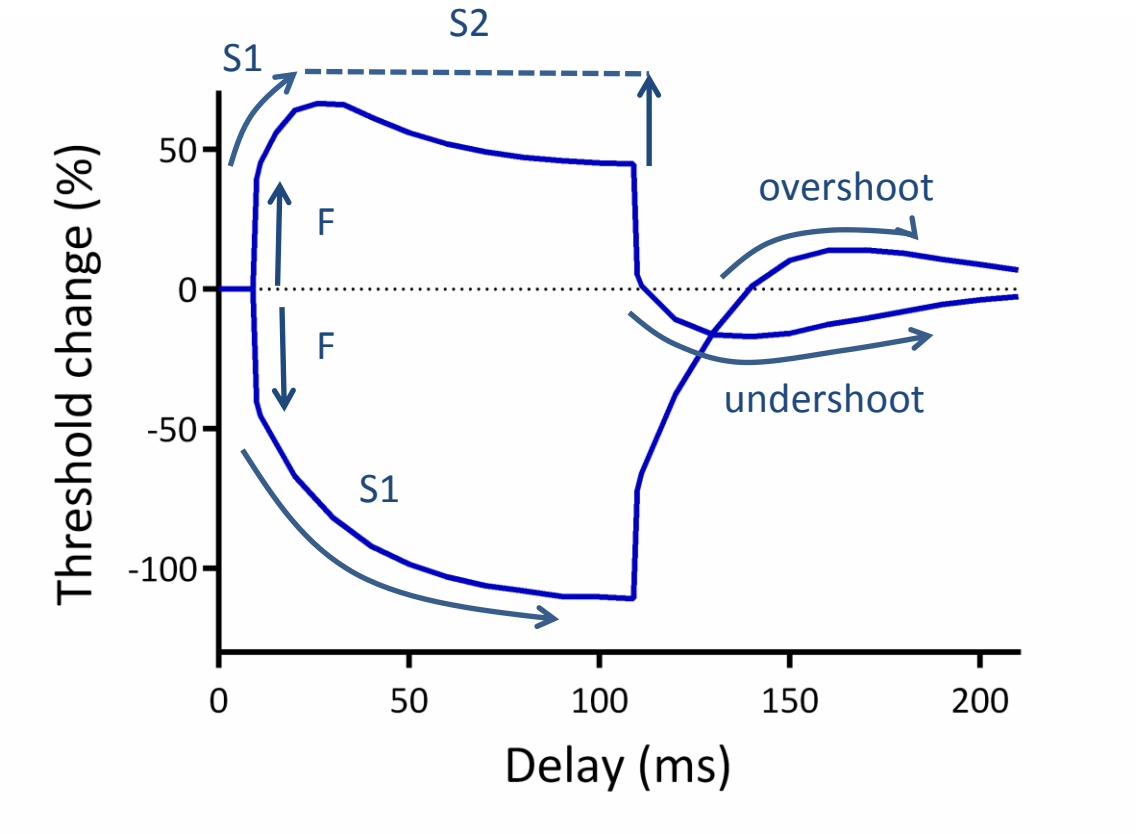

Figure 6. Threshold electrotonus waveform

Threshold electrotonus plot maps the changes in threshold during and after 100ms subthreshold depolarizing (plotted above baseline) and hyperpolarizing (plotted below baseline) conditioning pulses at ±40% threshold current. In response to depolarizing conditioning pulses (blue line), threshold first increases proportional to the applied current (fast phase or F phase). The activation of juxtaparanodal fast K^+ limits the extent of the S1 phase. Nodal slow K^+ currents ameliorate this increase in threshold, termed *accommodation*, and this is depicted by the S2 phase. The responses to hyperpolarizing pulses mirror these changes apart from exhibiting a more pronounced S1 phase. Ceasing the conditioning pulses returns threshold to baseline followed by a characteristic overshoot.

Current threshold relationship I/V relationship of axonal excitability investigates the relationship between long polarizing pulses and changes in threshold (Figure 7). It is analogous to the current voltage relationship and utilizes 200ms polarizing pulses to assess rectification properties of the axon (Kiernan et al., 2000). Polarizing currents are applied from +50% (depolarizing) and progressed by 10% steps through to -100% (hyperpolarizing). Steepening of the I/V curve in the depolarizing quadrant indicates greater outward rectification due to slow and fast K^+ currents (Kiernan et al., 2000). Steepening of responses to hyperpolarizing currents indicates inward rectification due to activation of hyperpolarization-activated I_h (Kiernan et al., 2000).

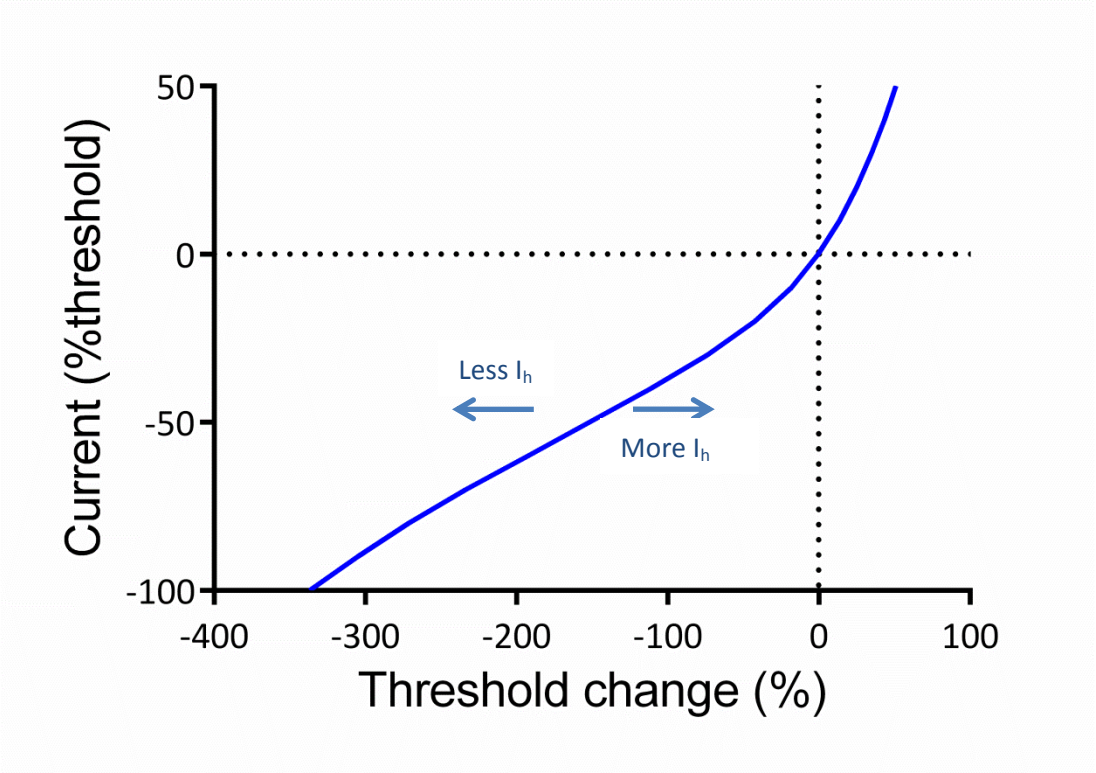

Figure 7. Current threshold relationship plot

Current threshold plot depicts changes in threshold after a 200ms depolarizing (upper right hand quadrant) and hyperpolarizing currents of (lower left hand quadrant). The relationship is analogous to the current voltage relationship and provides information on inward and outward rectifying conductances of the axon such that steepening of the curve indicates greater inward rectification (I_h).

Recovery cycle of axonal excitability analyses the change in threshold at varying interstimulus intervals after supramaximal pulses (Figure 8). Following an action potential, inactivation of Na_v channels render the axon in-excitable for 0.5-1ms (Hodgkin and Huxley, 1952), and this period is termed *absolute refractoriness*. When recovery of Na^+ conductances occurs, there is a period of relative refractoriness where action potential generation can only occur with currents higher than that of threshold (Kiernan et al., 2000). This typically lasts 3-4ms however, the periods can be affected by hyperpolarization and depolarization of the axonal membrane (Kiernan and Bostock, 2000). Following these periods of refractoriness, the axon undergoes a period of hyperexcitability where current threshold required to generate an impulse is lower than control impulses, termed *superexcitability* (Burke *et al.*, 2001, Krishnan *et al.*, 2009). Superexcitability is maximal at ~7ms and is related to the depolarizing afterpotential (Barrett and Barrett, 1982) whereby there is storage of charge on the internodal region, bringing the membrane potential closer to the threshold for activation. The extent of superexcitability is in part dependent on internodal membrane resistance specifically produced by paranodal K_v (Burke *et al.*, 2001). Following the period of superexcitability, the axon undergoes another period of reduced excitability termed *subexcitability*. Subexcitability occurs due to the slow closure of previously activated slow K_v, initiating hyperpolarization and thereby reducing excitability during this period.

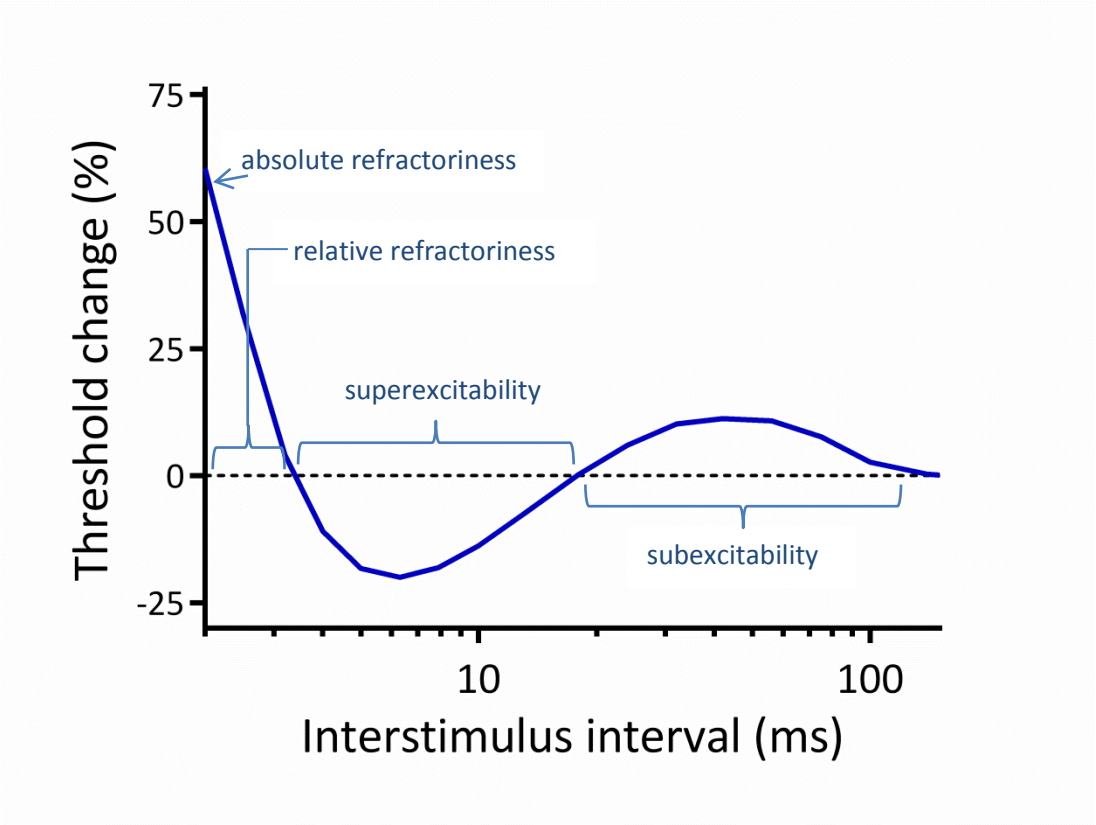

Figure 8. Recovery cycle waveform

This graph depicts the recovery of threshold change after a supramaximal stimulus at varying intervals (2-200ms). Reduced excitability is depicted above the baseline where greater threshold current is required to achieve target responses and vice versa for heightened excitability. Typical recovery of threshold after a supramaximal stimulus occurs in the order of: absolute refractoriness where the axon is in-excitable (dictated by inactivation of nodal Na_v); relative refractoriness where higher currents than threshold are required to produce the target response (dictated by recovery of Na_v); superexcitability , a period of heightened excitability where less current than threshold current elicits the target response (a result of capacitive charging of the internode and activity of paranodal K_v); subexcitability, a final period of reduced excitability dictated by activity of nodal slow K_v; and finally return to baseline excitability.

Axonal excitability: sources of variability

Reproducibility studies and variability between subjects- There have been only limited studies assessing the reproducibility of excitability recordings. Mogyoros and colleagues (2000) performed excitability assessments on median sensory afferents in 12 healthy subjects and compared within-subject variability of excitability parameters reflective of different nodal and internodal conductances, specifically refractoriness, SDTC and superexcitability (Mogyoros *et al*., 2000). These assessments were made at rest and with the application of background depolarizing currents to alter axonal membrane potential. The investigators reported a high degree of within- and between-subject variability in axonal excitability parameters concluding that single parameters alone may not be useful for making a clinical diagnosis. The authors concluded that the usefulness of the technique as a clinical tool would therefore be reliant on assessing internal consistency across multiple parameters.

Effect of temperature-Impulse transmission in large myelinated axons are dependent on temperature. Early reports detail reductions in conduction velocity and increased refractoriness with decreasing temperature (Johnson and Olsen, 1960, De Jesus *et al*., 1973, Lowitzsch *et al*., 1977) and decreases in compound sensory and motor action potentials (Ludin and Beyeler, 1977, Bolton *et al*., 1981). Later data on nerve conduction velocity demonstrated that the relationship with skin temperature was non-linear and most sensitive to change at lower temperatures (Todnem *et al*., 1989). The effect of temperature however on axonal excitability is less pronounced in the range of temperatures typically encountered in a clinical setting (Kiernan *et al*., 2001a). Burke and colleagues (1999) reported changes in sensory nerve excitability

parameters with a skin temperature reduction from 32^{o} to 22^{o} (Burke *et al*., 1999). This dramatic drop was associated with an increase in threshold of ~570%, indicating reduced propensity for action potential generation. A substantial increase in latency also occurred in the presence of lower temperatures. Notably however, the most prominent feature was a marked increase in refractoriness by 765%. Conversely, SDTC was minimally affected by temperature change. These findings were soon substantiated in motor studies (Mogyoros *et al*., 2000, Kiernan *et al*., 2001a) and extended to measurements of threshold (Kiernan *et al*., 2001a). The biophysical changes in response to temperature are thought to be due to changes in ion channel kinetics (Kiernan *et al*., 2001a). Significantly, the insensitivity of most excitability parameters to temperature change is explicable by their sole reliance on membrane potential which is only minimally affected by temperature (Kiernan *et al.*, 2001a).

Serum K^{+} concentration- Both intra- and extracellular K^{+} concentration are well established determinants of impulse generation and conduction (Hodgkin and Huxley, 1952). The evidence suggesting the effect of serum K^{+} on excitability parameters has been well reported in the setting of renal disease. Axonal excitability studies in end-stage kidney disease (ESKD) patients have reported changes consistent with membrane depolarization which has attributed to hyperkalaemia. As a means of eliminating the potential contribution of other serum solutes, Arnold *et al*. (2014) applied a serum K^{+} clamp during the dialysis sessions of four patients undergoing haemodialysis. K^{+} was clamped at 5mmol/L initially which was followed by the application of low dialysate K^{+} for the remainder of the session (Arnold *et al*., 2014). Median motor excitability was performed before, during and after the session. The axonal excitability abnormalities purported to occur in the presence of renal

disease were maintained during the period of clamping, only normalising after the serum K^+ had been dialysed to within normal levels. This study critically provided evidence for the effects of elevated serum K^+ on axonal function in human subjects. The excitability changes due to alterations in serum K^+ concentration have since been extended to healthy populations. A recent study by Boerio and colleagues (2014) indicated that excitability indices were responsive to changes in serum K^+ even within the normal physiological ranges of 3.1-4.7mmol/L (Boerio *et al*., 2014). Significant differences were noted between low normal (3.1-3.4mmol/L) and high values (4.3-4.7mmol/L) and that the direction of change in excitability was consistent with patterns reported for abnormally high serum K^+ in ESKD patients.

Limitations of axonal excitability techniques and alternative assessments

While axonal excitability techniques are useful tools in providing information regarding axonal ion channel function in the human setting (Krishnan *et al*., 2009), there are limitations which must be noted in interpretation in the patient context. With relation to the present thesis, a significant limitation of axonal excitability techniques is that their application is specific to assessing large myelinated fibres. A number of systemic disorders, including impaired glucose tolerance and diabetes mellitus may produce neurological symptoms that are reflective of dysfunction in small unmyelinated fibres (Malik *et al*., 2011, Lauria *et al*., 2012). These cannot be assessed using excitability techniques. For assessment of small fibre involvement in peripheral neuropathy, techniques that can be used include quantitative sensory testing (QST) and intraepidermal nerve fibre density (IENFD). QST is a form of psychophysical assessment that involves the application of graded noxious and innocuous stimuli both mechanical and thermal and recording responses (Backonja *et*

al., 2013). Perceptual thresholds are used as an indicator of the integrity of small fibres. QST is able to discriminate between disease states and healthy controls (Medici *et al*., 2013) and has been used extensively in pain research, providing a predictive measure of patient response to pharmacological intervention for neuropathic pain (Olesen *et al*., 2013). In recent years, reference values and standardised methodology for its application have been proposed (Backonja *et al*., 2013) which have provided a solid framework for utility of QST as a diagnostic measure. It is however recommended that QST be used in concert with intraepidermal nerve fibre density (IENFD) measures for diagnosing small fibre neuropathy (Krumova *et al*., 2012). IENFD requires a skin biopsy and quantitative measurement of nerve fibre density. It is considered a reliable tool with well-established normative values (England *et al*., 2009, Lauria *et al*., 2010). It is also sensitive to early pathology and a study comparing surrogate markers of small fibre neuropathy in diabetes indicated that IENFD and associated branch densities were lower in diabetic patients even in the absence of clinical small fibre neuropathy (Quattrini *et al*., 2007). Although a useful technique for direct measurement of small fibre pathology, a primary issue of IENFD studies is that the technique involves skin punch biopsies which are invasive (Quattrini *et al.*, 2007). This is problematic in long term monitoring which would involve repeat biopsy, promoting risk of infection.

Another limitation of axonal excitability recordings relates to the fact that these techniques are indirect markers of ion channel function. As such, alteration in the kinetics or function of a single ion channel has compensatory effects on the functions of other channels which can confound interpretation of excitability values. This issue

has been partially circumvented through the development of computer assisted mathematical modelling. The recent development of a validated mathematical model for human motor and sensory axons has assisted with interpretation of excitability data (Bostock *et al.,* 1991, Kiernan *et al.,* 2005, Howells *et al.,* 2012). The model consists of two space clamped compartments, namely a node and an internode, which are connected by pathways through and under the myelin sheath (Barrett and Barrett, 1982; Bostock *et al.* 1991). These have been based on results from animal studies analysing depolarizing afterpotentials, ionic conductances, clamp and latent addition studies in myelinated axons (Barrett and Barrett, 1982, Baker *et al.*, 1987, Bostock and Rothwell, 1997, Reid *et al.*,1999, Sholz *et al.,* 1993 and Schwarz *et al.,* 1995) and more recently supported by human studies of I_h and K^+ currents (Boerio *et al.*,2014, Howells *et al.,* 2012). Modelling has been successfully implemented in the interpretation of excitability results in other metabolic conditions including porphyria and chronic kidney disease (Lin *et al.*, 2008, Arnold *et al.*, 2014).

Another potential limitation of excitability techniques relates to the differences that may occur in conditions where there is superimposed structural change, as may occur in the development of clinical neuropathy. Typically, excitability techniques involve targeting 40% of maximal compound muscle action potential (CMAP)/compound sensory action potential and are thus only reflective of axons whose thresholds are recruited at that intensity of stimulus. A study by Shibuta and colleagues utilized targets of 10%, 40% and 60% in healthy individual and diabetic subjects to elucidate whether the excitability measures differ between different threshold populations. They found that tracking at lower targets and thus recruiting lower threshold populations, resulted in reduced changes in threshold in response to depolarizing and

hyperpolarizing stimuli, concluding that greater transient Na^+ currents occur in larger diameter fibre populations. The differences in biophysical properties between threshold populations of axons were confirmed by other investigators (Mori *et al.*, 2010, Trevillion *et al.*, 2007, Trevillion *et al.*, 2010), adding that recovery cycle changes were minimal and that there was a trend towards slightly higher SDTC in axon populations with lower thresholds. Analyses made at a single tracking level are therefore limited in determining the full scope of axonal function. Studies in disease cohorts of patients with DPN and amyotrophic lateral sclerosis (ALS) have demonstrated that when tracking axons with lower thresholds, the degree of membrane depolarization appears to be more pronounced (Shibuta *et al.*, 2010 and Shibuta *et al.*, 2013), suggesting that axons with lower thresholds for activation were more severely affected by the disease process. Consequently utilizing a single threshold level for assessing axonal pathology in disease may only provide a narrow perspective of underlying processes, a factor that should be considered in conditions where there is significant progressive axonal degeneration (Shibuta *et al.*, 2013, Mori *et al.*, 2010).

DIABETES MELLITUS

Diabetes Mellitus (diabetes) defines a state of hyperglycaemia due to reduced, altered or absent insulin secretion and or action. The diagnosis of diabetes in a clinic is made typically when glycosylated haemoglobin (HbA1c%) $\geq$ 6.5% . Additionally, elevated fasting plasma glucose (>7mmol/L), random blood glucose (>11.1mmol/L) and oral glucose tolerance test (>11mmol/L at two hrs) also confirm diagnosis (American Diabetes Association, 2010). In cases where type 1 diabetes requires confirmation, the presence of anti-GAD antibodies is assessed.

The chronic exposure to dysglycaemia, insulin deficiency and or insulin in patients with diabetes, facilitates the development of a number of systemic comorbidities including renal failure, retinopathy and neuropathy. On a societal level, diabetes and resulting comorbidities account for a cost of approximately AUD$10.6 billion (Lee *et al.*, 2013). This estimated value is inclusive of the cost for medication and adjuvant therapy directly targeting diabetes, government subsidies and cost to employers and businesses.

Pathophysiology

The history of diabetes is extensive, with early extant works of Arateus (80 – 138CE) documenting the sweet tasting urine of afflicted individuals. This finding was later more rigorously investigated by Dobson (1776) who recognized that elevated glucose could account for the sweetness. It wasn't until the works of Von Mering and Minkowski (1889) that the syndrome was connected to the pancreas, whereby dogs developed symptoms similar to humans after pranceatectomy (Mering and Minkowski 1890). Of the classical investigations, and relative to current treatment

and pathophysiological knowledge, the works of Banting and Best in the early 1920's pioneered treatment of diabetes by reversing the syndrome in experimental animal models with the administration of pancreatic islet cells (Banting *et al.*, 1991).

There are a number of aetiopathogenic mechanisms that underlie the diabetic state and it is these pathological mechanisms that separate the two primary types of diabetes.

Type 1 diabetes is characterized by a marked deficiency of insulin and generally is of acute clinical onset. Type 1 diabetes is characterized by the need to administer exogenous insulin from initial diagnosis (Tuomi *et al.*, 1993, Fourlanos *et al.*, 2005, Liu *et al.*, 2014). A recent Australian audit , demonstrated that 22.7% of patients that attended diabetes centres across Australia presented with type 1 diabetes (Australian Diabetes Educators Association, 2010). Type 1 diabetes is characterized by a specific autoimmune destruction of the Islets of Langerhans in the pancreas, which was first described in the early 1900's (Opie, 1901). Markers of this autoimmunity include antibodies to islet cells, anti-GAD antibodies and autoantibodies to tyrosine phosphatases 1A-2 and 1A-2β and can be used to confirm diagnosis of type 1 diabetes (American Diabetes Association, 2014). Specifically, destruction of *β-cells*, the insulin secreting cells of the pancreas, provokes the total loss of insulin and co-released C-peptide initiating the requirement for exogenous insulin.

This form of diabetes typically arises in early life but can also occur in older adults (>35 years of age) as a subtype of diabetes referred to as latent autoimmune diabetes of adulthood or *LADA*. LADA is further characterized by the presence of anti-GAD antibodies, which cross react with antigens on islet cells.

Type 2 diabetes –manifests with underlying resistence of biological tissues to insulin which portend a compensatory overproduction of insulin by pancreatic β-cells. Eventual failure of this system to maintain insulin output to manage the degree of insulin resistence occurs and elevated blood glucose levels ensue. This form of diabetes occurs in 85-90% of cases of diabetes in Australia and is most commonly associated with morbidity in the presence of *metabolic syndrome*: a metabolic state comprised of dysglycaemia, dyslipidaemia and elevated body mass index (BMI). Although classically appearing later in life, there has been an alarming upsurge of reported type 2 diabetes in children (Fazeli Farsani *et al.*, 2013) due to the growing incidence of obesity in youth populations (Patterson *et al.*, 2014). This earlier onset of type 2 diabetes in children is of major concern as micro and macrovascular complications are more pronounced earlier in life and thus early metabolic management is of crucial importance in this population (Wong *et al.*, 2015).

Treatment

Treatment of diabetes is focused not only on glycaemic control but management of co-morbidities and etiological factors (American Diabetes Association, 2013). Clinically, this is achieved through a combination of drug therapy, insulin supplementation and rigorous lifestyle management involving a number of health care professionals.

Type 1 diabetes management constitutes exogenous synthetic insulin therapy via two primary methods: multiple daily insulin injections (MDII) and insulin pump therapy or continuous subcutaneous insulin infusion (CSII) (Atkinson and Eisenbarth, 2001). MDII involves injecting a slow acting insulin analogue that delivers basal levels of insulin and a rapid acting analogue before meals for carbohydrate clearance with

food intake. A more recent advancement, CSII, utilizes fast acting insulin analogues that automatically perfuse insulin at a basal rate through a cannula placed subcutaneously.

Type 2 diabetes management targets not only hyperglycaemia but also other metabolic factors that are the essence of metabolic syndrome (Inzucchi *et al.*, 2012). The first approach involves lifestyle modification with recommendation from the American Diabetes Association including dietary advice, diabetes education and an emphasis on physical activity (Inzucchi *et al.*, 2012). Lifestyle changes promoting weight loss have been shown to have better efficacy than drug therapy for reducing incidence of type 2 diabetes (Diabetes Prevention Program Research Group, 2002).

Additional to weight loss and lifestyle changes, the administration of oral anti-hyperglycaemic agents (OHAs) are used to reduce hepatic glucose production (metformin/biguanides and thiazolidinediones) (Bailey and Turner, 1996, Lamanna *et al.*, 2011), increase insulin secretion (sulfonylurea insulin secretagogues, meglitinides and thiazolidinediones) (Yki-Jarvinen, 2004, Bryan *et al.*, 2005, Gerich *et al.*, 2005) and enhancing sensitivity of biological tissue to insulin (thiazolidinediones) (Gerich *et al.*, 2005).

Finally, insulin therapy may be advised in type 2 diabetes in the event of β-cell loss and resultant demise of appropriate insulin secretion. This partial loss dictates a less frugal insulin regimen compared to type 1 diabetic treatment (Cryer, 2002). In essence, insulin therapy is advised to create a normal glycaemic profile without overt weight gain (Bergenstal *et al.*, 2008), a side effect of insulin. Patients utilize typically longer or intermediate acting insulin analogues such as glargine/detemir or a neutral protamine Hagedorn (NPH) respectively (Inzucchi *et al.*, 2012). Only in more severe

or complicated cases whereby the above analogues are insufficient to maintain desired glycaemia, do type 2 diabetic patients require fast acting insulin analogues also (lispro and glulisine) (Inzucchi *et al.*, 2012).

Glycaemic variability

There is debate surrounding the appropriate method of assessing glycaemic control in patients (Brownlee and Hirsch, 2006). Since its introduction in 1977, glycosylated haemoglobin (HbA1c%) has been the primary means of assessing glycaemic control (International Expert Committee, 2009). However, there is gaining evidence that *glycaemic variability* is also an important measure of glucose exposure in diabetes (Brownlee and Hirsch, 2006) . Specifically, variability of blood glucose levels or *glycaemic variability* has been linked to oxidative stress (Monnier *et al.*, 2006), endothelial dysfunction (Hoffman *et al.*, 2013) and overall mortality in patients (Krinsley, 2008, Di Flaviani *et al.*, 2011). These metabolic events can be extensively screened by continuous glucose monitoring, whereby a sensor containing an assay is inserted subcutaneously, reacting with interstitial glucose and projects glucose readings using a mathematical algorithm and regular finger-stick glucose measurements for calibration (Klonoff, 2005). These measurements are indicated for reducing periods of hypoglycaemic events and have been utilised as a means of improving glycaemic control (Pickup *et al.*, 2011).

DIABETIC NEUROPATHY

The most common neurological complication of diabetes and the focus of this thesis is the development of a distal symmetric polyneuropathy (DPN) which affects ~50% of patients at long-term follow-up (Pirart, 1978, Dyck *et al.*, 1993). Patient morbidity and quality of life are profoundly impacted by DPN, with the presence of DPN accounting for 60-70% of diabetic foot ulceration and 50-75% of non-traumatic foot amputations (Gonzalez and Oley, 2000, Vinik *et al.*, 2006).

Clinical presentation and diagnosis

Patients with DPN typically present with symptoms such as hyperalgesia, allodynia, paraesthesias, burning and numbness in distal regions of the lower limbs, with symptoms later progressing proximally (Vileikyte *et al.*, 2003). Diagnosis of DPN is confirmed when NCS are abnormal and symptoms of distal symmetric polyneuropathy are present (Tesfaye *et al.*, 2010). However, lower limb NCS abnormalities are considered a prerequisite for diagnosis (Kohara, Kimura et al. 2000, Dyck, O'Brien et al. 2005).

However, composite scores including NCS have been suggested for assessing and diagnosing DPN, showing greater sensitivity to progression of the disease (Dyck *et al.*, 2005). England et al. (2009) suggested that diagnoses of distal symmetric polyneuropathy be based on clinical symptoms, signs and NCS abnormalities (England *et al.*, 2009). Composite scores for staging severity of neuropathy in DPN have therefore come into existence. One such score is the Total Neuropathy Score (TNS).The score incorporates subjective symptom report, assessment of sensation (vibration and pinprick) and reflex testing, all staged in length dependent manner i.e.

more severe scores reflect more proximal distributions of signs and symptoms. Additionally, sural and tibial NCS are also incorporated. The TNS was initially validated in a cohort of patients with DPN (Cornblath *et al.*, 1999) and its application has been extended to staging neurotoxicity of chemotherapy treatment (Griffith *et al.*, 2010).

Pathogenesis and biophysical abnormalities in animal models of DPN

Animal models of DPN have demonstrated prominent changes in axonal function (Craner *et al.*, 2002, Hong *et al.*, 2004, Hong and Wiley, 2006). The development of DPN is currently viewed as occurring in two separate phases, namely an initial reversible stage that is responsive to intervention and a second stage of irreversible nerve injury that is characterized by nerve conduction slowing and a reduction in action potential amplitude (Sugimoto *et al.*, 2000, Arnold *et al.*, 2013).

During the reversible stage, there are prominent changes in axonal biophysical properties that may be amenable to pharmacological intervention (Kamiya *et al.*, 2004). Of note, reduced function of the energy-dependent axonal Na^+/K^+ pump has long been recognised as a potential contributor to the development of DPN (Graf *et al.*, 1981, Greene and Lattimer, 1984, Greene, 1986). Altered Na^+/K^+ pump activity may be due to C-peptide deficiency (Wahren *et al.*, 2000, Sima *et al.*, 2004) or metabolic alterations resulting from hyperglycaemia. These include the formation of advanced glycation end products (AGE) which initiate oxidative stress and upregulation of the polyol pathway, causing an increase in intra-axonal sorbitol levels and subsequent myoinositol depletion (Greene *et al.*, 1988). These changes in turn result in a deleterious reduction in protein kinase C activation (Zhu and Eichberg, 1990) which is required for Na^+/K^+ pump function. Reduced Na^+/K^+ pump

activity has also been postulated to cause structural changes in nodal and paranodal regions of the axon, where there exists a high density of voltage-gated Na^+ channels (Sima and Brismar, 1985). More recently, altered expression of voltage-gated Na^+ channels and associated mRNA have been found in animal models of DPN and these results have suggested that Na^+ channel dysregulation may play a role in the development of neuropathic pain in DPN (Craner *et al.*, 2002, Hong *et al.*, 2004). Specifically, modification of Na^+ channels by phosphorylation and methylglyoxal, a reactive dicarbonyl formed in the presence of hyperglycaemia (Thornalley, 2005), have been linked to increased firing of nociceptive neurones and heightened pain states in diabetic mice models (Hong *et al.*, 2004, Bierhaus *et al.*, 2012).

Issues with clinical neurophysiological techniques in DPN

While a wide range of therapeutic approaches have been proposed for the treatment of DPN, including antioxidants, aldose reductase inhibitors and nerve growth factors (Habib and Brannagan, 2010), clinical results have been disappointing. One of the major hurdles that these clinical trials have faced is the absence of an objective functional test that permits the detection of mild neuropathy (Vinik *et al.*, 2005). Standard techniques, such as NCS and quantitative sensory testing, are based on the detection of axonal loss, which renders them unsuitable for early diagnosis and, consequently, for prevention of disability (Vinik *et al.*, 2005). As a result, DPN frequently remains undiagnosed until there is irreversible nerve injury, which may lead to foot infection, ulceration and even amputation (Veves *et al.*, 1993, Manes *et al.*, 2002).

Alterations in axonal excitability in DPN: links to pathogenesis

There have been numerous recent studies of axonal excitability, undertaken in both type 1 and type 2 diabetic patients, and these have provided further support for the role of biophysical changes as important contributors to the development of DPN (For review see Arnold 2013). Excitability studies in DPN have demonstrated significant changes in parameters of threshold electrotonus and the recovery cycle (Kitano *et al.*, 2004, Krishnan and Kiernan, 2005, Misawa *et al.*, 2006, Sung *et al.*, 2012). The first comprehensive study of axonal excitability in DPN was undertaken by Kitano and colleagues (2004) who assessed median motor axonal excitability in a cohort of 21 diabetic patients, comprised of patients with both type 1 and type 2 diabetes. Enrolled patients had poorly controlled diabetes with a mean HbA1c% of 10.9% (normal <6.5%) (Gillett, 2009). Baseline excitability studies demonstrated reductions in refractoriness, duration of the relative refractory period and strength-duration time constant (SDTC), findings that were interpreted as evidence of reduced trans-axonal Na^+ gradients. Patients were then re-assessed following a four week period of intensive insulin treatment. Post-treatment excitability studies demonstrated prominent improvements in Na^+ channel dependent parameters, with an increase in refractoriness and strength-duration time constant as well as improvement in standard NCS parameters including median nerve conduction velocity and F wave latency. The authors concluded that hyperglycaemia was associated with a reduced trans-axonal Na^+ gradient, which was subsequently restored with strict glycaemic control. These conclusions were supported by further studies that demonstrated a significant relationship between hyperglycaemia and the presence of excitability abnormalities (Misawa *et al.*, 2005). In a cohort of 79 patients, those with poor glycaemic control (HbA1c% >9) had significantly shorter SDTC values compared to

patients with better glycaemic control (HbA1c% < 7%) (Misawa *et al.*, 2005). A similar reduction in Na^+ conductances was suggested by a second study of 58 diabetic patients, in which poor glycaemic control was associated with shorter relative refractory periods (Misawa *et al.*, 2004). The authors interpreted these findings as evidence that poor glycaemic control may reduce inward Na^+ conductances.

The potential benefits of pharmacological intervention in restoring Na^+ channel conductances in DPN was further emphasised in a study of 30 diabetic patients enrolled in a clinical trial of epalrestat, an aldose reductase inhibitor that is postulated to improve nerve conduction by reducing the conversion of glucose to sorbitol (Misawa *et al.*, 2006). In addition to routine excitability assessment, studies were also undertaken using the technique of latent addition, which assesses both passive input membrane properties and persistent Na^+ conductances (Bostock and Rothwell, 1997). Baseline excitability abnormalities again suggested changes in Na^+ conductances, with reductions in refractoriness and SDTC. Following pharmacological treatment, changes in latent addition were noted that suggested an increase in persistent Na^+ conductances. In total, these studies provided strong evidence of altered Na^+ conductances in human diabetic nerve and demonstrated that these changes are amenable to pharmacological intervention.

Aside from alterations in Na^+ channel properties, animal studies of DPN have also demonstrated alterations in function of the energy-dependent axonal Na^+/K^+ pump (Greene and Lattimer, 1984). These studies have suggested that altered Na^+/K^+ pump function may occur due to C-peptide deficiency (Wahren *et al.*, 2000) and shunting of glucose through the polyol pathway, leading to increased levels of sorbitol and

depletion of myoinositol (Nakamura *et al.*, 1999, Vincent *et al.*, 2004, Edwards *et al.*, 2008). A reduction in myoinositol was postulated to cause reduced protein kinase C activation, which is necessary for Na^+/K^+ pump function (Zhu and Eichberg, 1990). The possibility that Na^+/K^+ pump dysfunction may play a role in the development of DPN was supported by a study of median axonal excitability, undertaken in 20 DPN patients. Excitability recordings demonstrated prominent changes in threshold electrotonus parameters, with reduced changes to depolarizing and hyperpolarizing currents leading to a 'fanned-in' appearance of the threshold curve(Krishnan and Kiernan, 2005). The changes in threshold electrotonus were more severe in patients with more advanced grade of neuropathy. Similarly, a fanning in of the recovery cycle curve was also noted in the patient cohort. This pattern of change is consistent with axonal membrane depolarization, which may occur in the setting of Na^+/K^+ pump dysfunction due to intracellular retention of Na^+ ions leading to an excess of positive charge within the axon (Kiernan and Bostock, 2000). It was concluded that these changes were not related to the previously described alterations in Na^+ conductances but represented a discrete biophysical abnormality that reflected axonal energy insufficiency in diabetic axons (Krishnan and Kiernan, 2005).

Other studies have specifically explored the role of Na^+/K^+ pump dysfunction in DPN using two different dynamic manoeuvres, namely limb ischaemia and maximal voluntary muscle contraction (MVC). Ischaemia alters membrane potential by paralysing energy-dependent processes, principally the Na^+/K^+ pump, while MVC leads to an influx of Na^+ ions during the contraction followed by a heightened level of Na^+/K^+ pump activity after cessation of contraction. With both ischaemia and MVC, excitability changes during and after the period of intervention have been used

as surrogate markers of Na^+/K^+ pump function (Vagg *et al.*, 1998, Krishnan and Kiernan, 2005). Studies of nerve ischaemia in DPN demonstrated reduced changes in excitability parameters during the ischaemic period when compared to normal control data, a phenomenon that has been termed 'ischaemic resistance' (Weigl *et al.*, 1989, Strupp *et al.*, 1990). The cause of the reduced changes with nerve ischaemia remains unclear and a number of different explanations have been put forward including Na^+/K^+ pump dysfunction (Kuwabara *et al.*, 2002), altered anaerobic glycolysis in diabetic axons (Strupp *et al.*, 1990) and the presence of membrane depolarization with a consequent reduction in axonal metabolic demand (Weigl *et al.*, 1989). Studies of MVC showed reduced magnitude of post-contraction excitability changes in patients with overt DPN and a slower return of excitability parameters to pre-contraction baseline values, when compared to both diabetic subjects without neuropathy and age-matched normal controls (Krishnan *et al.*, 2008). Moreover, these studies demonstrated activity-dependent conduction block in a subset of DPN patients, with a significant correlation between the degree of block and baseline level of refractoriness (Krishnan *et al.*, 2008). Patients who had the greatest reductions in refractoriness at baseline, possible reflecting reduced nodal Na^+ conductances, appeared to be predisposed to the development of conduction failure following a period of natural activity. These findings raised the possibility that biophysical abnormalities in DPN may contribute to the development of motor symptoms of DPN such as weakness and fatigue.

The contribution of biophysical changes to the development of neuropathic symptoms has been explored using excitability techniques, initially in healthy control subjects (Mogyoros *et al.*, 1997) and later in disease cohorts (Misawa *et al.*, 2009). In

particular, studies in control subjects have demonstrated the important contribution of persistent Na^+ conductances to the development of ectopic sensory symptoms (Mogyoros *et al.*, 1997). The changes noted are yet to be explored in terms of their relationship to neuropathic symptoms and subsequent effect on quality of life in DPN patients.

Axonal excitability as a predictive biomarker in DPN

One of the major hurdles in developing treatments for DPN is the absence of sensitive clinical markers that can provide early detection of axonal dysfunction (Vinik *et al.*, 2005). The sensitivity of axonal excitability assessments in detecting alterations in axonal function has provided a basis for studies exploring early alterations in nerve function that are undetectable by standard clinical methods. In a large cross- sectional study of type 2 diabetic patients, Sung and colleagues (2012) reported a reduced threshold change in threshold electrotonus and recovery cycle parameters, similar to those reported previously (Krishnan and Kiernan, 2005, Misawa *et al.*, 2005). Importantly, these reductions became progressively more prominent with increasing neuropathy severity indicating that excitability assessment may be sensitive in detecting progressive changes in axonal function in DPN (Sung *et al.*, 2012). This study also found that the patients without clinical or nerve conduction evidence of neuropathy had reduced threshold change in depolarizing threshold electrotonus and superexcitability when compared to an age-matched control population. Similar findings were noted in a study undertaken by Bae and colleagues (2011) in which 'fanning-in' of the threshold electrotonus curve, increased refractoriness and reductions in superexcitability and subexcitability were noted in patients with early DPN. This study also demonstrated an association between excitability values and both triglyceride levels and estimated glomerular filtration rate, suggesting that axonal dysfunction in type 2 diabetes may be related to other metabolic factors apart from hyperglycaemia alone (Tesfaye *et al.*, 2005).

Recent studies have extended these insights into a type 1 diabetic population. Arnold and colleagues (2013) assessed excitability parameters in 20 type 1 diabetic patients,

in whom the presence of neuropathy had been excluded using clinical and nerve conduction criteria. They demonstrated reductions in depolarizing threshold electrotonus at multiple time points, as well as alterations in recovery cycle parameters that suggested axonal membrane depolarization. The changes were significant when compared to both non-neuropathic type 2 diabetic patients, matched for glycaemic control, as well as age-matched controls. In addition to demonstrating the potential utility of excitability testing in detecting early axonal dysfunction, this study also provided evidence that the heightened predisposition of type 1 diabetic to neuropathy development (Sima, 2003) may lie in differences in axonal biophysical properties and that these alterations may be due to the combined effects of . insulinopenia and C-peptide deficiency (Sima, 2003, Sima *et al.*, 2004).

Methodology

Recruitment of diabetic patients

All patients were recruited through the Diabetes Centre at the Prince of Wales Hospital. Patients with signs of carpal tunnel syndrome and who were taking neuropathic medications were excluded from the study. Patients involved in the study had been diagnosed for and receiving their respective treatments for a minimum of 12 months.

The entire patient cohort consisted of both type 1and type 2 diabetic patients who had been diagnosed as per standard clinical diagnosis utilizing a combination of:

1) Elevated HbA1c% (≥6.5%)
2) Fasting plasma glucose (>7mmol/L)
3) Random blood glucose (>11.1mmol/L)
4) Oral glucose tolerance test (>11mmol/L at 2hrs)
5) Presence of anti-GAD antibodies and C-peptide deficiencies (confirm diagnosis of type 1 diabetes)

All subjects gave written informed consent in accordance with the Declaration of Helsinki, and the study was approved by the South Eastern Sydney Area Health Service and the University of New South Wales Research Ethics committees.

Recruitment and testing of control subjects

All subjects gave written informed consent in accordance with the Declaration of Helsinki, and the study was approved by the South Eastern Sydney Area Health Service and the University of New South Wales Research Ethics committees. Control subjects were recruited through the Prince of Wales Hospital and the University of New South Wales Australia. All normal controls underwent clinical

screening to exclude neuropathy. Control subjects underwent neurophysiological testing (i.e. excitability testing) under the same conditions as the patients (temperature and setup) and were matched for age and sex to disease cohorts.

Equipment

- Neurotip disposable needles (Owen Mumford Ltd., Oxford, UK) for assessing pinprick sensation.
- 128 Hz tuning fork used to measure vibration sense.
- Clinical reflex hammer to test tendon reflexes.
- Medelec Synergy EMG system (Oxford Instruments, Old Woking, UK) used for NCS testing and recording.
- Medelec AC amplifier (AA6 MKIII) (Medelec, Surrey, UK) used to amplify excitability recordings.
- Data acquisition device (DAQ PCI-6221); Shielded connector block(BNC-2110); Cable (SHC-68-68-EPM); (National Instruments, Austin, USA) used to convert recordings to a digital signal.
- Isolated linear bipolar constant current stimulator (DS5, Digitimer, Welwyn Garden City, UK).
- Hum Bug 50/60 Hz Noise Eliminator(Quest Scientific Instruments, North Vancouver, Canada) used to cancel background electrical noise.
- Automated threshold tracking software - QTRAC (Institute of Neurology, Queen Square, London) with TRONDNF axonal excitability protocol.
- Conventional non-polarizable ECG electrodes (Unilect 7831Q; Unomedical, Stonehouse, Great Britain) to provide surface stimulation/reference and for surface recordings.
- Surface EMG recording electrodes (Kendall Soft-E, H69P, Tyco Healthcare, Gosport, UK).
- Electrosurgical neutral electrode (Unilect 2406M, Unomedical, Stonehouse, Great Britain) used as ground electrodes.
- Red Dot Trace prep (2236, 3M Canada) for abrading skin in preparation for electrode placement.
- Thermistor thermometer (5831-A, OmegaEngineering, Manchester, UK) used to measure skin temperature.
- Guardian Real-Time CGM system (Medtronic Minimed, Northridge, CA) used to obtain continuous glucose readings.

- ACCU-CHEK Performa Glucometer (Roche Diagnostics,Mannheim, Germany) for measuring on the spot blood glucose levels obtained by finger-stick.
- Enlite sensor and Minilink transmitter (Medtronic MiniMed Inc., Northridge, CA, USA).

Axonal excitability

Median axonal excitability in this thesis was assessed using the TRONDNF protocol applied by QTRAC automated software (Digitimer, London, UK) as previously published (Kiernan *et al.*, 2001b). Specifically, testing utilized surface stimulation and was performed by placing an active electrode over the median nerve, 4-6cm proximal to the crease at the wrist, and an anode placed on bony point 10-15cm proximal and lateral to stimulation point. Stimulation was performed using a DS5 Isolated Bipolar Constant Current Stimulator (Digitimer, England) and test pulses for motor and sensory assessments were 1 and 0.5ms respectively for all test paradigms.

For motor axonal assessments, recordings were obtained from abductor pollicis brevis (APB) using non-polarizable ECG electrodes placed over the motor point of APB. A reference electrode was placed on the proximal phalanx of the same hand (Figure 9a)

For sensory recordings, antidromic responses at digit II of the same hand was detected using a ring electrodes, one placed between the metacarpophalangeal and the proximal interphalangeal joint and the other on the distal phalangeal joint. The ground electrode for recording was typically placed on the palmar surface of the hand for both motor and sensory recordings (Figure 9b).

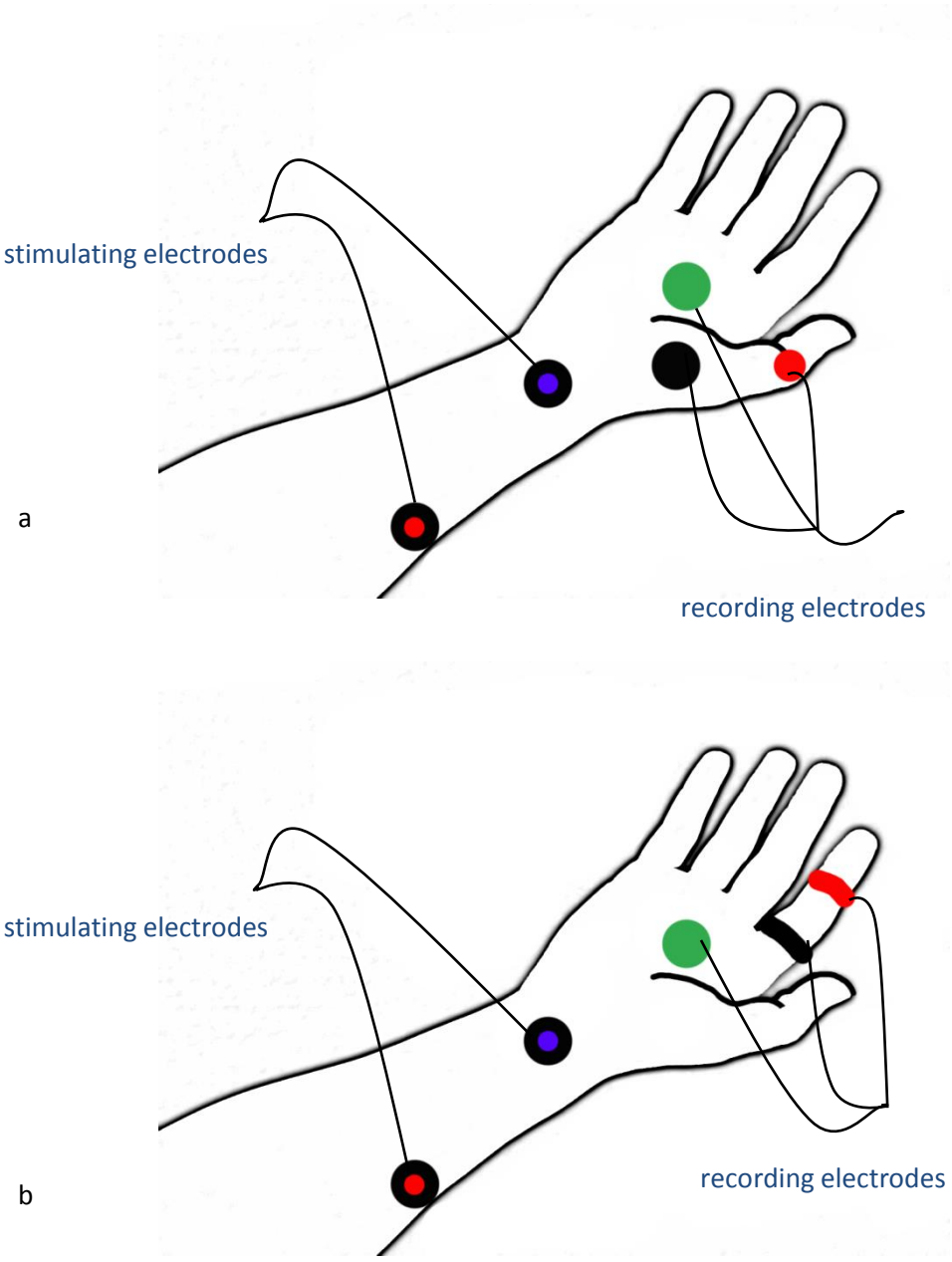

Figure 9. Axonal excitability setup for testing the median nerve

Stimulation was applied at the wrist with cathode placement ~4cm proximal to the crease of the wrist (black circle with blue) and the anode placed offset from the median nerve on a bony landmark (black circle with red). For motor studies (a) recordings were taken from APB (black) with a reference electrode placed over the bone of the phalanx (red). For sensory recordings (b), the ring electrodes were placed on digit II, with recording electrode (black) placed proximal to the reference electrode (red). Earth electrode was placed typically on the palm (green) for both motor and sensory studies.

QTRAC software (Institute of Neurology, Queen Square, UK) allowed for fast acquisition of axonal excitability properties relating to five distinct testing paradigms: Stimulus Response (SR) behaviour, strength-duration-time-constants (SDTC), threshold electrotonus (TE), current threshold relationships (I/V) and the recovery cycle (RC) (see Assessment of axonal function in the clinical setting).

SR curves were obtained using a 1 ms (motor assessments) or a 0.5ms (sensory assessments) duration stimulus. Current was applied in 2% increasing steps until the maximal CMAP or sensory nerve action potential (SNAP) response was established. CMAP responses were measured from baseline to peak and SNAP responses were measured from peak to peak of the recorded waveform. At each level of current increase, the average of three responses was taken. In the remaining tests, a target of ~ 40% of this maximum response was utilized and the stimulus required to achieve this target was termed "threshold" (Figure 4).

Strength-duration relationship was established by applying Weiss' Law as the relationship between strength and duration of a stimulus using four stimulus durations: 0.2, 0.4, 0.8, 1ms for motor and 0.1, 0.2, 0.4 and 0.5ms for sensory recordings. The strength-duration time constant (SDTC) is reflective of nodal persistent Na^+ conductances and is defined as the x intercept of the relationship between stimulus charge and width whilst Rheobase is defined as the gradient of this relationship (Figure 5).

Threshold electrotonus was determined by plotting the percentage of threshold change when test pulses were applied during and after 100ms sub-threshold conditioning currents of +40% (depolarizing - TEd) and -40% (hyperpolarizing - TEh) control threshold current (note for Chapter 3, additional measurements using ±

20% conditioning currents were used) (Figure 6). A total of 26 time points were assessed and more than 10 parameters are acquired from this paradigm.

In response to TEd and TEh, parameters are determined from the average percentage threshold change between 10-20ms, 20-40 and 90-100ms. After cessation of conditioning currents, TEd(undershoot) and TEh(overshoot) are calculated as the mean value of maximal threshold reduction in the 20ms following cessation of the conditioning stimulus. Parameters calculated from responses to TEd also include peak threshold change during the S1 phase of depolarizing threshold electrotonus (TEdpeak) and S2 Accommodation which is defined as the difference between TEdpeak and TEd(90-100ms).Threshold electrotonus provided information on internodal properties and overall axonal membrane potential.

Current threshold relationship (I/V) was determined by mapping change in threshold when test impulses were delivered at the end of a 200ms depolarizing and hyperpolarizing conditioning currents (+50 to – 100ms) stepped in 10% increments (Figure 7). I/V provides information regarding rectification properties of the internode (Pape, 1996, Kiernan et al., 2000). Three parameters were obtained from the I/V paradigm: resting I/V (calculated from polarizing currents -10% to 10%), minimum I/V (minimum slope obtained by fitting a straight line between consecutive points) and hyperpolarizing I/V (computed from the three maximal hyperpolarized conditioning currents).

The *recovery cycle* assessed the change in threshold that occurs over 200ms following supramaximal stimulation. It provides information on Na^+ and K^+ channels at the nodal and paranodal regions of the axon (Kiernan et al., 2000). The change in threshold was assessed at 18 test-intervals (2-200ms).

The recovery cycle of excitability undergoes a discrete and predictable series of changes (Figure 8). This first is refractoriness. In the TROND protocol this is summarized by a) percentage change in threshold required at 2.5ms post supramaximal stimulus and b) the period where threshold change is returning to baseline, termed relative refractory period (RRP). Following this period is a period of heightened excitability termed superexcitability. This was calculated as the mean threshold change of the three maximal points in this period. This period is followed by subexcitability, a period of reduced excitability whereby greater stimuli are required to elicit the target response. This was quantified as the mean percentage change of the three maximal adjacent points during this phase.

For all excitability recordings performed on both patients and control subjects, skin temperature was measured at the site of stimulation for the duration of the test using a thermister thermometer (5831-A, OmegaEngineering, Manchester, UK). Subject skin temperature was maintained at $\geq 32^{o}$C. In instances where subject skin temperature fell below 32^{o}C, a heat pack or blanket was applied in order to increase the temperature. Testing was then recommenced following a 5-10 minute period of acclimatisation.

Nerve conduction studies

Nerve conduction studies (NCS) were performed as part of the clinical neurological assessments in all patients. Testing suites involved a combination of sural and tibial NCS as per standard neurophysiology protocols (Kimura, 1983), assessing conduction velocities and amplitudes. All studies were performed using a Medelec Synergy EMG system (Oxford Instruments, Old Woking, UK) and surface EMG recording electrodes (Kendall Soft-E, H69P, Tyco Healthcare, Gosport, UK). Tibial nerves were assessed by stimulating between the medial malleolus and calcaneal tendon and recording from abductor hallucis. Sural nerves were assessed by stimulating ~12 cm proximal to the ankle on the calf and recording at the lateral malleolus (Kimura, 1983). Grounding during sural recordings was achieved using a lead electrode. NCS findings were used to determine the presence of DPN and for use in the Total Neuropathy Score to grade severity of neuropathy (see *Staging neuropathy severity* below)

Staging neuropathy severity

Comprehensive neurological assessment was conducted in all patients and neuropathy severity was graded using a revised version of the Total Neuropathy Score (TNS) which in its original form has been validated for use in diabetic subjects (Cornblath *et al.*, 1999). The revised version has been previously used to stage neuropathy in excitability studies of diabetic patients (Sung *et al.*, 2012).

The revised TNS is a composite score of 8 categories (Figure 10). The categories included the extent and severity of sensory and motor symptoms, assessment of deep tendon reflexes, muscle strength, vibration sensibility (128 Hz tuning fork), pinprick

sensibility (Neurotip™, Owen Mumford, United Kingdom) and tibial and sural nerve amplitudes (Medelec Synergy system, Oxford Instruments, United Kingdom). Each category was scored from 0 – 4 (0 = no dysfunction, 4 = severe dysfunction) and summed to give a total score from 0-32 (32 = maximum dysfunction). The TNS was further subdivided into total neuropathy grades (TNG) to indicate severity of neuropathy; TNG 0 = TNS 0-1, TNG 1 =TNS 2-8, TNG 2 = TNS 9- 16, TNG 3 = TNS 17 -24 and TNG 4 = TNS 25 -32 (Cornblath *et al.*, 1999). For the purpose of the studies in this thesis TNG 0 is referred to as "no neuropathy", TNG 1 as "mild neuropathy", TNG 2 as "moderate neuropathy", TNG 3 as "severe neuropathy" and TNG 4 as "very severe neuropathy".

Assessment of neuropathy-specific quality of life

Impairment in QoL was assessed using the Neuropathy Specific Quality of Life Questionnaire (NeuroQoL) (Figure 11). The NeuroQoL assess symptom emotional burden and restrictions on activities of daily living specific to DPN (Vileikyte *et al.*, 2003). It has been used to measure the impact of DPN on QoL (Davies *et al.*, 2006) and for assessment of the effectiveness of potential neuropathic treatments (Lavery *et al.*, 2008). Importantly, NeuroQoL has been shown to discriminate more clearly between varying severities of DN, compared to more generic QoL scales such as the SF-36 (Vileikyte and Boulton, 2000) and SF-12 (Vileikyte *et al.*, 2003). The questionnaire consists of 35 questions and rates the effect of neuropathy on QoL as a product of their presence and total level of bother. In the present study, the following domains were assessed: 1) symptomatic affliction 2) psychosocial impairment 3) DPN specific impact 4) overall QoL. The average rating for the questions in domains

1-3 were summed to give a total NeuroQoL impairment score of 3-35 (3 = nil QoL impairment from neuropathy, 35= maximal QoL impairment from neuropathy).

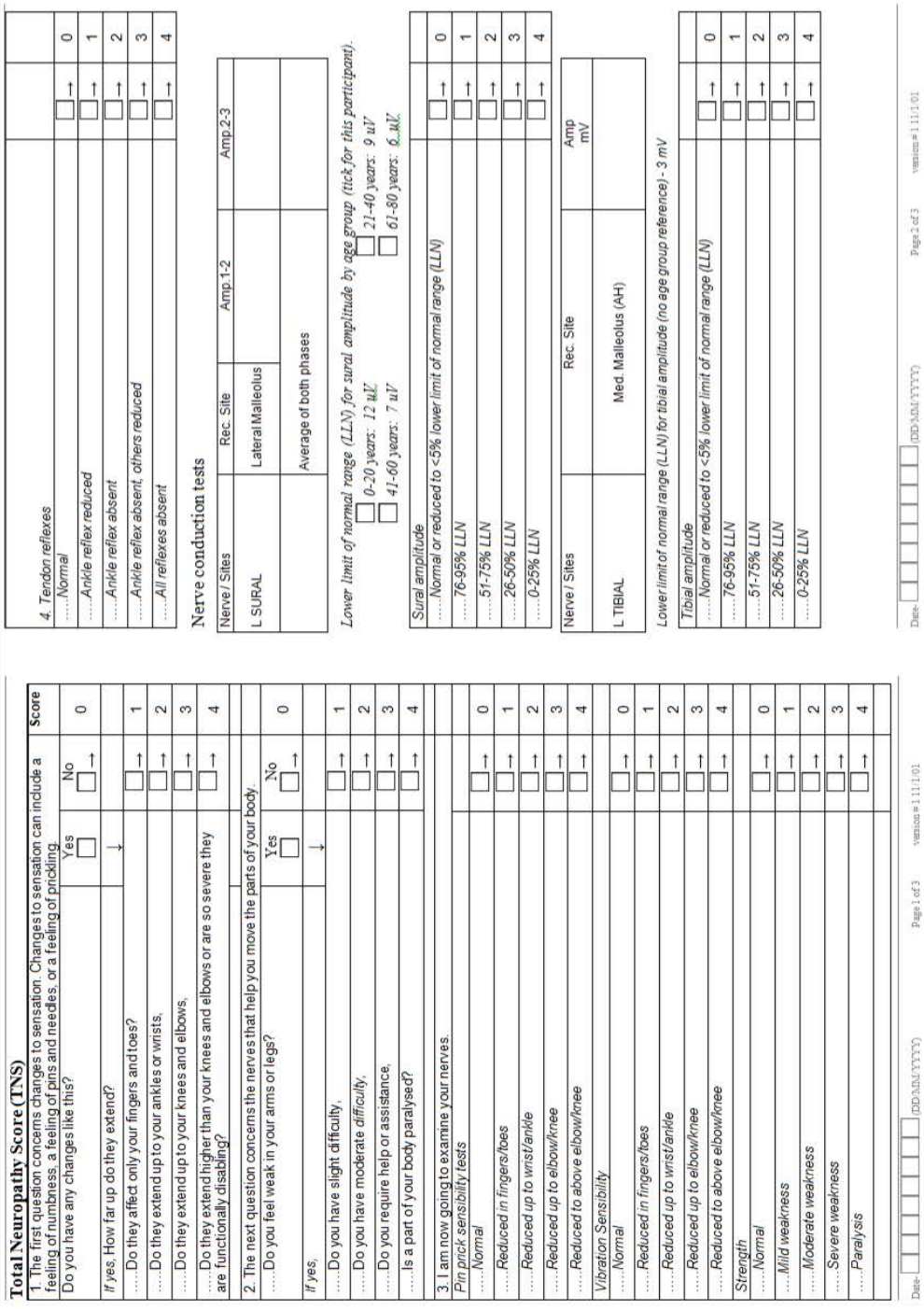

Figure 10. Total Neuropathy Score

The revised version of the Total Neuropathy Score (TNS) has been adapted from the original TNS (Cornblath *et al.*, 1999). It provides a composite score based on standard clinical neurological assessments including NCS. *Figure is a reproduction of the revised version of the TNS*

In the past 4 weeks how often have you experienced the following symptoms?	All the time	Most of the time	Some of the time	Occasionally	Never	**How much bother did this cause you?** Very much	Some bother	None
1. Burning in your legs or feet								
2. Excessive heat or cold in your legs or feet								
3. Pins and needles in your legs or feet								
4. Shooting or stabbing pain in your legs or feet								
5. Throbbing in your legs or feet								
6. Sensations in your legs or feet that make them jump								
7. Irritation of the skin caused by something touching your feet, such as bedsheets or socks								

A. Have these **painful symptoms** reduced your quality of life?	Very much	Quite a lot	Somewhat	A little	Not at all

In the past 4 weeks how often have you experienced the following symptoms?	All the time	Most of the time	Some of the time	Occasionally	Never	**How much bother did this cause you?** Very much	Some bother	None
8. Numbness in your feet								
9. Inability to feel the difference between hot and cold with your feet								
10. Inability to feel objects with your feet								

B. Have these **last three symptoms** reduced your quality of life?	Very much	Quite a lot	Somewhat	A little	Not at all

Figure 11. Neuropathy specific quality of life questionnaire sample

The Neuropathy specific quality of life questionnaire (NeuroQoL) is a validated tool for assessing impact of DPN on multiple domains of QoL (symptomatic, psychosocial, DPN specific and overall QoL). The questionnaire graded symptoms in terms of frequency and level of bother (Vileikyte *et al.*, 2003). *Figure is only a partial reproduction of the full NeuroQoL*

Continuous glucose monitoring

Continuous glucose monitoring was used to determine glycaemic variability in patients. The system utilized was a Guardian REAL-Time Continuous Glucose Monitoring system with Medtronic Enlite Sensors. Sensors were inserted subcutaneously as per standard usage, at least 10cm from the umbilicus and or insulin pump infusion site depending on patient preference and comfort (Figure 12). The cannula of the sensor draws interstitial fluid samples for reaction with the sensor assay. Glucose levels are determined by the electrochemical reaction between glucose in the interstitial fluid from the sample and the assay which is corrected to blood sample calibrations made by the patient. Utilizing a Minilink transmitter (MiniMed, CA) the sensor assay readings were projected to either the Guardian Monitor or to the patient's pump (Medtronic Paradigm Veo). Finger-stick measurements of blood glucose levels were taken every 3-4 hours to calibrate the sensor and projections of blood glucose levels were performed by the system.

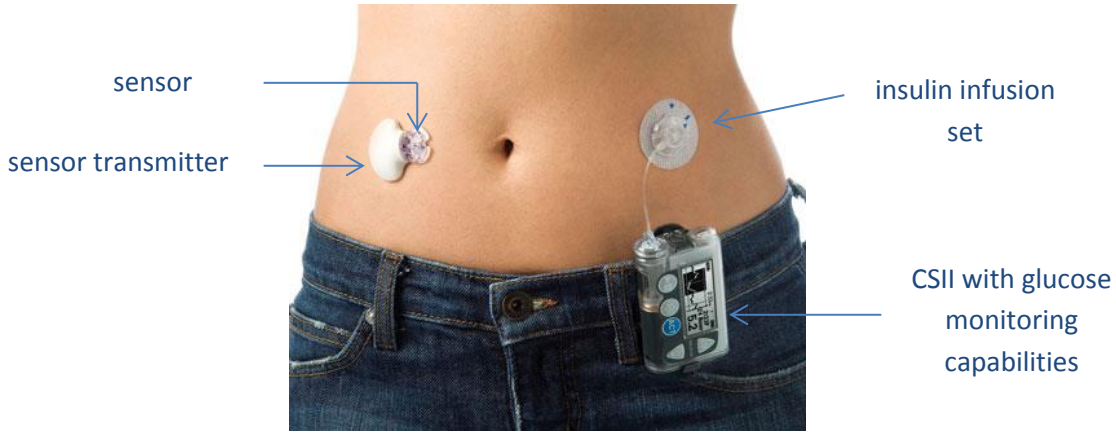

Figure 12. Continuous glucose monitoring system and continuous subcutaneous insulin infusion set

Continuous glucose monitoring system utilizes an interstitial glucose sensor implant and wireless transmitter (*Medtronic*). Interstitial fluid is drawn via a subcutaneous cannula into an assay well and projected glucose values are calculated. Readings are displayed on either a

continuous glucose monitor or continuous subcutaneous insulin infusion set (CSII). *Figure was adapted from an image taken from Medtronic (Northbridge, CA, USA)*

Glycaemic variability calculations

Glycaemic variability calculations were made as per the methods originally described by Service and colleagues (1970) and used extensively in studies of glycaemic variability in patients (Monnier *et al.*, 2006, Monnier *et al.*, 2007, Di Flaviani *et al.*, 2011). Specifically, mean amplitude of glycaemic excursions (MAGE) was calculated from two consecutive 24 hour periods of continuous glucose monitoring. Excursions of glucose either peak to trough (nadir) or nadir to peak that was greater than 1 standard deviation of the two days' blood glucose recordings were averaged.

Mathematical modelling

The effect of type 1 diabetes on human motor and sensory axons was examined using the 'Bostock' model of axonal excitability (Bostock *et al.*, 1991, Howells *et al.*, 2012). This model focuses on axonal properties that govern the nodal excitability of human axons as measured using axonal excitability techniques. The model consists of two space clamped compartments, namely a node and an internode, which are connected by pathways through and under the myelin sheath (Barrett & Barrett, 1982; Bostock *et al.* 1991). Voltage-gated fast and slow K^+ conductances were modelled at both the node and the internode, while persistent and transient Na^+ conductances were only present on the nodal membrane. Capacitive leak and pump currents across the axolemma were modelled separately for the node and internode.

The model was adjusted to fit mean control data separately for motor and sensory axons before fitting to the corresponding diabetes mean data. The model fitting followed an iterative least squares approach where the overall discrepancy was

assessed and minimized using the weighted sum of the squares of the error terms between the modelled and group data.

Statistical analysis

Statistical analysis was performed using SPSS statistics software v. 20 (IBM, Chicago, IL).Normality of data was determined using Shapiro-wilk test. Following this, for comparison of group mean data (i.e. patient data vs normal control data and between treatment groups), either Student's t-tests or Mann Whitney U tests were performed for parametric and non –parametric data respectively. Correlations between neurophysiological parameters and clinical characteristics either Pearsons correlations or Spearman Rho correlations, were undertaken dependent on normality of data. Cross-sectional analysis between different groups were made using a one way ANOVA with Bonferroni post hoc tests to establish between which groups there were differences, whilst comparison of patient data under progressive treatment was undertaken using repeated measures ANOVA or mixed model analysis if there were missing data points. Statistical significance was considered when $P<0.05$.

Chapter 1 – Neurophysiological parameters and their relationship to neuropathy specific quality of life in type 2 diabetes mellitus.

SUMMARY

Pharmacological agents for neuropathic pain management in DPN target a number of mechanisms including Na^+ channel function and gamma-alpha-buteric-acid (GABA)-minergic processes. At present, prescription is undertaken on a trial-and-error basis, leading to prolonged medication trials and greater healthcare costs. Axonal excitability techniques are a novel method of assessing axonal ion channel function in the clinical setting. The aim of study was to determine the effects of axonal ion channel dysfunction in DPN on neuropathy-specific quality of life (QoL) measures.

Fifty-four patients with type 2 diabetes underwent comprehensive neurological assessment, NCS and axonal excitability assessment. Neuropathy severity was assessed using the Total Neuropathy Score. Neuropathy-specific-QoL was assessed using the neuropathy-specific-QoL questionnaire (NeuroQoL). HbA1c% and BMI were recorded in all patients.

NeuroQoL scores indicated significant QoL impairment (mean=9.08$\pm$5.93). Strength-duration time constant (SDTC), an excitability parameter reflecting Na^+ channel function, was strongly correlated with QoL scores (r=0.545, P<0.005). SDTC was prolonged in 48.6% of patients who experienced neuropathic symptoms. A significant correlation was also noted between SDTC and neuropathy severity (r = 0.29, P <0.05). This relationship was strengthened when looking specifically at patients with clinically graded neuropathy (r = 0.366, P <0.05).

The present study has demonstrated an association between markers of Na^+ channel function and QoL in DPN. The study demonstrates that excitability techniques may

identify patients in who altered Na^+ channel function may be the dominant abnormality. The findings suggest that excitability techniques may have a role in clinical decision making regarding neuropathic pain management.

INTRODUCTION

Diabetic peripheral neuropathy (DPN) is a frequent complication of diabetes affecting 30-50% of patients with type 2 diabetes (Australian Diabetes Educators Association, 2010). DPN manifests as a length dependent polyneuropathy and as such, patients typically present with sensory symptoms such as pain, paraesthesia and numbness in distal lower limb segments which may progress to motor and upper limb involvement in more severe cases (Sugimoto *et al.*, 2000).

It is well established that quality of life (QoL) is significantly impaired in DPN patients, affecting physical, social and emotional wellbeing (Quattrini and Tesfaye, 2003, Vileikyte *et al.*, 2003). Currently, treatment options for the neuropathic symptoms of DPN include anticonvulsant medications that have effects on voltage-gated ion channels, gamma-aminobutyric acid (GABA)-mimetic agents, and selective noradrenaline and serotonin reuptake inhibitors. These medications either block Na^+ channels or alter the balance between inhibitory and excitatory neurotransmitters, reducing peripheral and central sensitization (Maneuf *et al.*, 2001, Lenkey *et al.*, 2010). At present, the selection of a specific drug for an individual patient is largely undertaken on a trial and error basis (O'Connor, 2009, Yarnitsky *et al.*, 2012), frequently leading to prolonged trials of medication, development of significant side effects and greater health care costs (Vinik, 1999, Yarnitsky *et al.*, 2012).

Previously, human studies have demonstrated that ectopic sensory symptoms such as paraesthesia and pain may be related to changes in axonal ion channel properties (Mogyoros *et al.*, 1997). Specifically, these studies have provided evidence that

axonal persistent Na^+ channels may play a major role in the generation of ectopic symptoms, both sensory and motor (Mogyoros *et al.*, 1996). These insights have been established through the use of axonal excitability testing. Previous studies utilizing nerve excitability techniques in patients with diabetes have demonstrated prominent alterations in axonal ion channel properties, including changes in parameters reflecting Na^+ channel function (Misawa *et al.*, 2004, Krishnan and Kiernan, 2005, Misawa *et al.*, 2009). While these changes in ion channel properties have been postulated to underlie the development of neuropathic symptoms (Krishnan *et al.*, 2008), the relationship between these changes and QoL in a patient cohort with diabetes has not been evaluated. The aim of the present study was to investigate the potential relationship between axonal ion channel dysfunction, ectopic sensory symptoms and QoL measures in a cohort of patients with type 2 diabetes.

METHODS

Eighty –seven patients with type 2 diabetes mellitus were consecutively recruited from the Diabetes Centre at Prince of Wales Hospital in Sydney. Exclusion criteria for this study were current treatment with neuropathic medications, clinical or electrophysiological features of carpal tunnel syndrome and a history of neuropathic symptoms for less than 6 months. On the basis of these criteria, 54 patients (M28:F26) underwent motor axonal excitability studies.

Neuropathy severity was determined using a revised version of the Total Neuropathy Score (TNS) (Cornblath *et al.*, 1999) (as per Methodology). Neuropathy severity was graded according to the TNS:TNG 0 = 0-1, TNG 1 =2-8, TNG 2= 9- 16, TNG 3 = 17 -24 and TNG 4 = 25 -32 (Cornblath *et al.*, 1999). For the purpose of this study we refer to TNG 0 as "no neuropathy", TNG 1 as "mild neuropathy", TNG 2 as "moderate neuropathy", TNG 3 as "severe neuropathy" and TNG 4 as "very severe neuropathy". QoL was assessed using the NeuroQoL(see *Methodology*) and included assessment of the effect of ectopic symptoms on QoL four domains 1) symptomatic affliction 2) psychosocial impairment 3) DPN specific impact 4) overall QoL.

Motor axonal excitability studies were recorded from the median nerve. The following excitability parameters were recorded using the previously described TRONDNF protocol as described in *Methodology*: SDTC, an axonal excitability marker reflective of nodal persistent Na^+ conductances (Bostock and Rothwell, 1997) which are thought to underlie the generation of ectopic neuropathic symptoms (Bostock, 1983, Mogyoros *et al.*, 1996); recovery cycle parameters superexcitability, subexcitability and RRP; and threshold electrotonus parameter S2 Accommodation.

Cumulatively, these recovery cycle and threshold electrotonus parameters were used as markers of membrane potential (Kiernan and Bostock, 2000).

Comparisons were made between patient data and normal controls using Student's t-tests or Mann Whitney U Tests were conducted where appropriate. To compare between TNG groups, one-way analysis of variance (ANOVA) or Kruskal Wallis tests depending on normality. Following this, the Bonferroni post hoc tests were applied to establish where differences occurred between groups. Finally, to determine the relationships between clinical characteristics, axonal excitability parameters and NeuroQoL scores, Spearman Rho correlations (two tailed) were conducted.

RESULTS

Clinical characteristics and Neuropathy grading

Of the 87 patients with type 2 diabetes who were screened, 54 were enrolled in the study (Figure 1. 1. Screening process for enrolment and testing: type 2 diabetic patients). As a group, the enrolled patients had an average age of 62.19yrs±1.28, BMI of 31.26±0.81kg/m^2, duration of diabetes for 147.32±15.53 months and HbA1c% of 7.98±0.23%. Mean characteristics per TNG are expressed in Table 1. 1. Clinical characteristics with increasing neuropathy severity: type 2 diabetic patients.

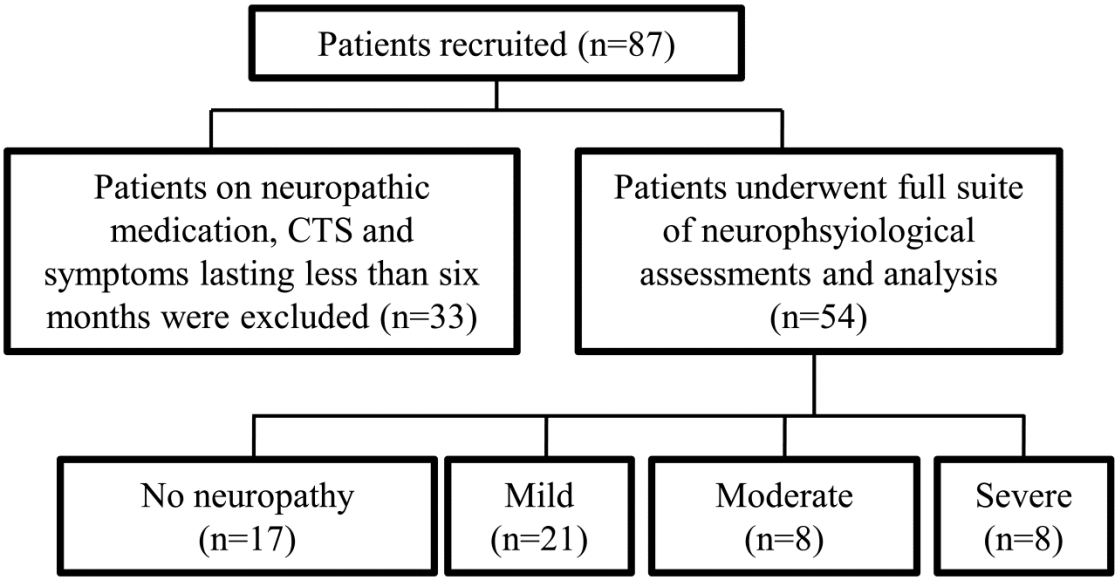

Figure 1. 1. Screening process for enrolment and testing: type 2 diabetic patients

Thirty-three patients were excluded based on existence of neuropathic pain medication usage, carpal tunnel syndrome and those with symptoms lasting less than 6 months. Fifty-four patients remained for further testing and neuropathy grading.

Table 1. 1. Clinical characteristics with increasing neuropathy severity: type 2 diabetic patients

Neuropathy severity	*no neuropathy* *n = 17*	*Mild* *n = 21*	*moderate* *n = 8*	*severe* *n = 8*
Percentage of total cohort	31.5%	38.9%	14.8%	14.8%
Age (yrs)*	58.82±2.0	59.38±1.85	67.88±2.55	71.00±3.14
Disease duration (months)	111.21 ± 20.80	12.72 ± 21.06	216.17 ± 63.36	214.25 ± 42.39
HbA1c%	6.88 ± 0.36	8.33 ± 0.35	7.9 ± 0.36	8.55 ± 0.63
BMI	31.03 ± 1.19	31.63 ± 1.60	30.59 ± 2.3	31.85 ± 1.92
Sural nerve amplitude (uV)**	11.83 ± 1.44	7.69 ± 1.63	3.16 ± 1.48	ABSENT
Tibial nerve amplitude (mV)**	11.09 ± 1.41	8.22 ± 1.07	3.84 ± 1.11	0.31 ± 0.63
NeuroQoL scores				
1) Symptomatic*	NA	2.25 ± 0.37	2.98 ± 1.06	4.84 ± 0.99
2) Psychosocial	NA	2.77 ± 0.71	2.39 ± 1.0	3.83 ± 0.49
3) DPN specific impact*	NA	1.68 ± 0.30	1.43 ± 0.43	3.13 ± 0.48
4) Overall QoL score	NA	2.16 ± 0.29	2.57 ± 0.20	3.13 ± 0.35
5) Total NeuroQoL score	NA	8.77 ± 1.77	9.45 ± 2.20	14.93 ± 1.52

All values given as mean ± SEM. n is given in brackets where data is missing. No neuropathy = TNG 0, mild = TNG 1, moderate = TNG 2 and severe neuropathy = TNG 3. Significant differences in parameters across severity groups are indicated as * $P<0.05$ and ** $P<0.005$.

Clinical neurological examination demonstrated a wide range of neurological impairment, with TNS scores ranging from 0–23, with a mean TNS score of 7.00±1.02 (Cornblath *et al.*, 1999). Further grading of patients according to TNS revealed that 31.5% of the cohort had no neuropathy (TNG 0), 38.9% had mild neuropathy (TNG 1) and 29.6% had moderate to severe neuropathy (TNG 2-3). Ectopic sensory neurological symptoms such as paraesthesia were reported in 53.7% of patients. Duration of diabetes (DOD) was correlated with increasing neuropathy severity ($r = 0.427$, $P < 0.001$). There were no significant correlations between BMI and HbA1c% with neuropathy severity (TNS scores).

Neuropathy specific Quality of Life findings

The Neuropathy-specific quality of life questionnaire (NeuroQoL) (Vileikyte *et al.*, 2003) was administered to patients with evidence of clinical neuropathy (TNG 1-3, n = 37) (Table 1. 1. Clinical characteristics with increasing neuropathy severity: type 2 diabetic patients). Overall NeuroQoL scores in this group ranged from 4 – 26.25, with a mean of 10.33 ± 1.0. The NeuroQoL was further divided into symptomatic, psychosocial and DPN specific scores, all of which showed impairment (Symptomatic score: 2.07 ± 0.14; Psychosocial score: 2.28 ± 0.18 and DPN specific score: 1.88 ± 0.22). Significant correlations were noted between TNS scores and Overall QoL, as well as symptomatic and psychosocial scores ($r = 0.61$: $P < 0.001$, $r = 0.44$: $P < 0.05$ and $r = 0.445$: $P < 0.05$ respectively).

Significant differences were also observed in mean NeuroQoL domain scores across the different severity groups. Specifically, symptomatic NeuroQoL scores were significantly higher in patients with severe neuropathy compared to patients with

mild neuropathy (mild: 2.25 ±0.37, severe: 4.84 ± 0.99, $P<0.05$). Psychosocial scores also demonstrated greater impairment in patients with severe neuropathy (0.383 ± 0.49), compared to those with mild (2.77 ± 0.71, $P<0.005$) and moderate neuropathy (2.39 ± 1.01, $P<0.005$). Similarly, DPN-specific QoL impairment was higher in the severe group (3.13 ± 0.48) compared to those in the mild (1.68 ± 0.30, $P<0.005$) and moderate neuropathy group (1.14 ± 0.43, $P < 0.05$).

Axonal excitability abnormalities

Prominent changes in excitability parameters were noted in DPN patients compared to normal controls (Figure 1. 2a) and these changes were progressively greater with increasing neuropathy severity (Figure 1. 2b). With increasing neuropathy severity, there was a reduction in mean CMAP amplitude (mV), with significant differences noted between normal controls and patients with severe neuropathy (controls, 6.56; severe group, 4.66 ± 1.18; $P < 0.05$). Distal motor latency also increased with greater neuropathy severity (TNG: r = 0.485, $P< 0.005$; TNS: r = 0.431, $P < 0.01$). SDTC, a neurophysiological parameter reflecting the behaviour of persistent Na^+ conductances (Bostock, 1983, Mogyoros *et al.*, 1996), was increased in patients with moderate to severe neuropathy when compared to normal controls (TNG2-3, 0.50 ± 0.02; controls, 0.44 ± 0.01; $P<0.05$).

The changes in SDTC were accompanied by changes in other excitability parameters that were qualitatively similar to those reported in previous studies of patients with diabetes (Krishnan and Kiernan, 2005, Misawa *et al.*, 2005, Misawa *et al.*, 2009) (Figure 1. 2). Progressive alterations were noted in the recovery cycle parameters

superexcitability ($P < 0.05$), late subexcitability ($P < 0.005$) and relative recovery period (RRP) in addition to the threshold electrotonus parameter S2 Accommodation($P < 0.005$) (Figure 1. 2). Taken together, these changes are consistent with axonal membrane depolarization, a finding that has been demonstrated in previous excitability studies of DPN patients (Krishnan and Kiernan, 2005, Sung *et al.*, 2012).

Correlations between axonal excitability abnormalities and neuropathy-specific QoL

Correlations were undertaken to explore the potential relationship between excitability parameters and QoL measures. A strong positive relationship was observed between strength-duration time constant (SDTC), a marker of the activity of persistent Na^+ conductances (Mogyoros *et al.*, 1996) and QoL impairment as a result of neuropathic symptoms (symptomatic NeuroQoL score: r = 0.545, $P<0.005$) (Figure 1. 3). There was a significant correlation between SDTC and total NeuroQoL score (r=0.515, $P<0.05$).Of the patients who experienced neuropathic symptoms, SDTC was prolonged in 48.6% of this group (upper limit 95% confidence interval = 0.462 ms). In addition, a positive correlation was noted between SDTC and increasing neuropathy severity (n=54, r = 0.29, $P <0.05$). This relationship was strengthened when looking specifically at patients with clinically graded neuropathy (n=37, r = 0.37, $P <0.05$).

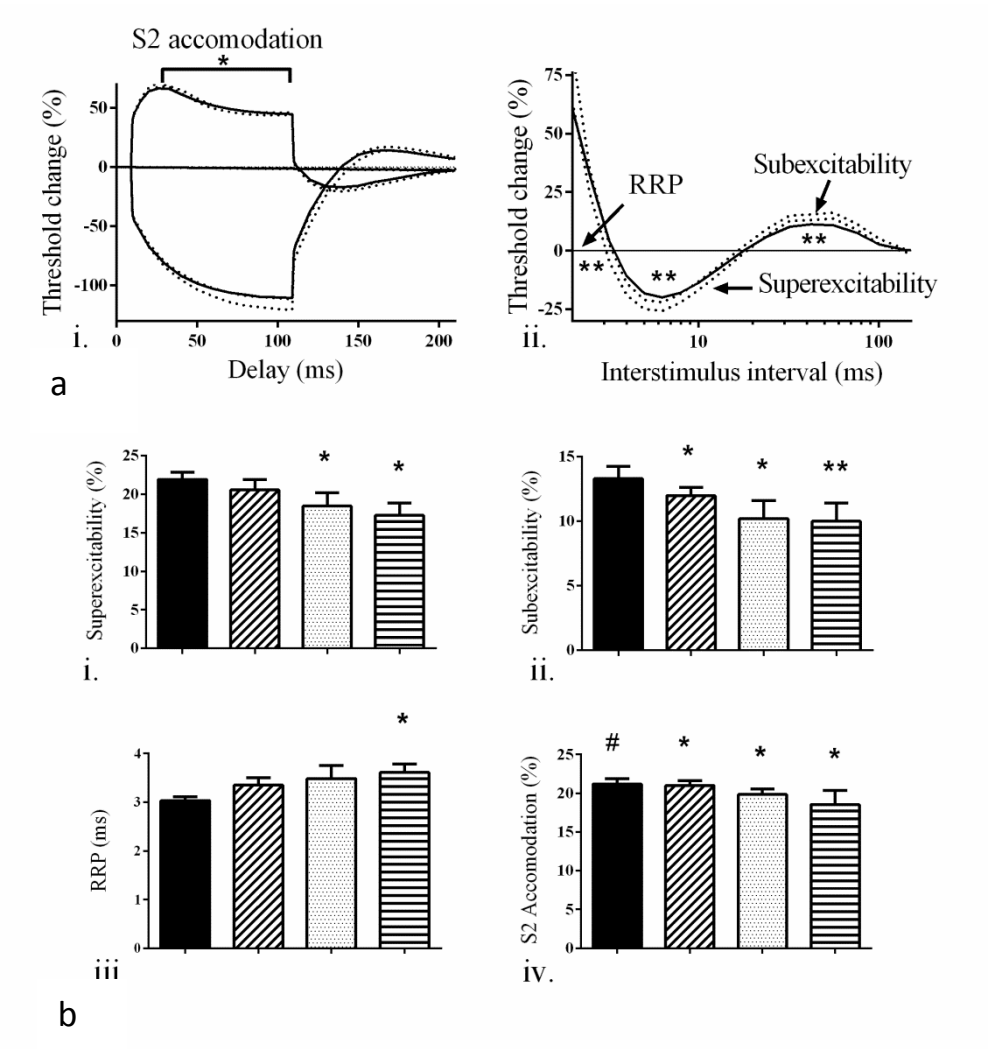

Figure 1. 2. Median motor excitability comparisons: type 2 diabetic patients vs controls and between neuropathy severity groups.

Figure 1.2a. Median motor excitability plots: type 2 diabetic patients vs controls. Patient mean data (block lines) were abnormal across multiple time points . i) threshold electrotonus and ii) recovery cycle curves. 95% confidence limits of normal controls are denoted by dotted lines. Mean superexcitability, subexcitability (RRP) and S2 Accommodation were found to be significantly different between patients and controls: * = P<0.05, ** = P <0.005.

Figure 1.2b. Comparison of excitability parameters with increasing neuropathy severity. Mean values ± SEM expressed: Block fill = patients with no neuropathy; diagonal line fill = mild neuropathy; dotted fill = moderate neuropathy and horizontal line fill = severe

neuropathy. Patients were compared to normal control data (not shown). Superexcitability (i), subexcitability (ii) and S2 Accommodation (iv) are expressed as percentage (%) change of threshold. Control mean: S2 Accommodation = 22.84 $\pm$ 0.50, superexcitability = 23.81$\pm$1.1 and subexcitability = 14.75 $\pm$ 0.60). The relative refractory period (RRP) (iii) is expressed in ms (controls: 3.028 ms). Taken together, these parameters indicate a progressive shift towards a more depolarized axonal membrane potential in the diabetic cohort.

In addition, correlations were undertaken between SDTC and symptoms of nerve hyperexcitability (i.e. paraesthesia and burning). A strong correlation was noted between increasing SDTC and symptoms of hyperexcitability ($r=0.745$; $P<0.05$). Further analysis demonstrated a weaker correlation between the occurrence of negative sensory symptoms (i.e. numbness and loss of sensation) and SDTC ($r=0.539$, $P<0.05$). There was no significant correlation between SDTC and symptoms of unsteadiness or impaired balance. Similarly, psychosocial, DPN-specific and Overall QoL scores were not significantly correlated with SDTC.

Analyses were also undertaken between NeuroQoL scores and other excitability measures that were not specifically related to Na^+ channel function, namely threshold electrotonus parameters and superexcitability. There was no significant correlation between QoL and these other neurophysiological parameters. In total, the findings suggest that the correlation between SDTC and QoL scores was not due to a generalised disturbance in axonal function but more likely reflected a specific effect of diabetes on persistent Na^+ conductances.

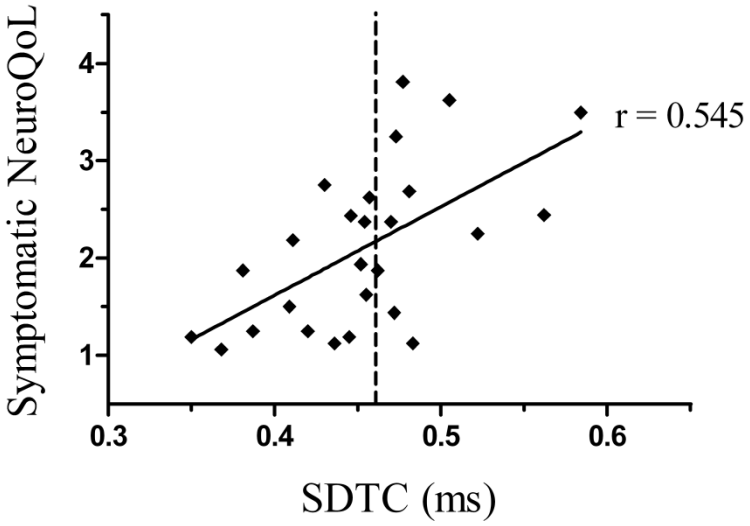

Figure 1. 3. Correlation between strength-duration-time-constant (SDTC) and symptomatic NeuroQoL score

SDTC strongly correlated with symptomatic NeuroQoL scores: r=0.55, $P<0.05$. Dotted line indicates the upper limit of normal range for SDTC of control cohort (upper 95% confidence limit: 0.46ms). SDTC was prolonged in 48.6% of symptomatic DPN patients compared to normal controls.

DISCUSSION

The present study has demonstrated an association between clinical markers of Na^+ channel dysfunction and QoL measures in a cohort of patients with DPN. Specifically, the study has shown correlations between strength-duration-time-constant, a parameter reflecting upregulation of nodal persistent Na^+ conductances(Bostock and Rothwell, 1997), and symptomatic neuropathy-specific QoL. These correlations were even stronger when analysis was limited to symptoms of nerve hyperexcitability. The importance of this association lies in the prominent causal role that has been attributed to nodal persistent Na^+ conductances in generating ectopic impulse activity in the peripheral nervous system (Bostock, 1983, Mogyoros *et al.*, 1997).

It may be argued that the underlying cause of the prolongations in SDTC may relate to age related changes in excitability (Jankelowitz *et al.*, 2007). However, the direction of change in SDTC (i.e. prolongation) is the opposite of that which occurs with age related change. Thus, the prolongation in SDTC appears to be independent of age related changes. Furthermore, the potential contribution of axonal membrane depolarization could be argued as a mechanism SDTC lengthening (Krishnan and Kiernan, 2005, Misawa *et al.*, 2009), given the pattern of change noted in threshold electrotonus and recovery cycle parameters with increasing neuropathy severity. Critically however, there was no correlation between other excitability parameters that reflect changes in membrane potential and neuropathy-specific QoL measures, suggesting that the correlation between SDTC and neuropathy-specific QoL were intrinsically related to upregulation of persistent Na^+ conductances rather than a more generalised change in axonal function. It is arguable also that the SDTC changes may

also be due to alteration in passive nodal conductances (Bostock and Rothwell, 1997). An investigation by Misawa and colleagues in diabetic subjects utilized *latent addition*, a technique which is capable of distinguishing between passive nodal and persistent Na^+ conductances. They demonstrated that the slow component of latent addition, a measure reflecting persistent Na^+ conductances , was strongly correlated with changes in SDTC (Misawa *et al.*, 2006). Their findings suggest that the prolongation in SDTC in diabetic patients is not related to passive nodal properties but rather represents an intrinsic change in persistent Na^+ conductances. While the present study focussed on upper limb motor recordings, the findings provide further evidence that excitability changes in diabetes may occur in a generalised distribution, despite the clinical features of DPN being largely lower limb predominant (Krishnan and Kiernan, 2005, Sung *et al.*, 2012).

Our findings suggest that persistent Na^+ conductances may be upregulated in patients with DPN. These findings are supported by investigations undertaken in animal studies which have demonstrated that increased Na^+ channel expression may play an important role in the generation of diabetic neuropathic symptoms (Hong *et al.*, 2004). Specifically, these studies have shown that there is upregulation of Na^+ channel isoforms in diabetic nerve and that these changes are associated with the development of neuropathic symptoms (Hong *et al.*, 2004). The increase in Na^+ currents has been attributed to Na^+ channel phosphorylation, which modifies channel properties and may cause an increase in channel conductance (Hong *et al.*, 2004). Studies specifically exploring the contributions of persistent Na^+ currents to diabetic neuropathic pain have demonstrated increases in mRNA levels for Na^+ channel isoforms (Craner *et al.*, 2002, Hong and Wiley, 2006). Such changes have been

postulated to result from alterations in signal transduction cascades and the increased levels of protein kinase A and protein kinase C induced by diabetes, which further enhance Na^+ channel expression and ectopic firing (Craner *et al.*, 2002).

Persistent Na^+ conductances play an important role in the generation of ectopic neuropathic symptoms and a number of neuropathic pain treatments preferentially modulate persistent Na^+ conductances (Spadoni *et al.*, 2002, Lenkey *et al.*, 2011). In the present study, 48.6% of patients with neuropathic symptoms had prolonged SDTC, consistent with an upregulation of persistent Na^+ conductances, while the remainder had a normal or reduced SDTC. This finding suggests that the contribution of altered Na^+ channel function towards the development of neuropathic symptoms may vary between individual patients. In patients with a normal SDTC, it is possible that other mechanisms may play a greater role in the development of neuropathic symptoms, including reduced descending inhibition to pain (Maneuf *et al.*, 2001, Maneuf *et al.*, 2004), altered levels of excitatory neurotransmitters (Cardoso *et al.*, 2011) or increased sensitization of central pain related areas (Rose and Kam, 2002, Fischer *et al.*, 2009). These mechanisms may not cause changes in voltage-gated ion channel function of peripheral nerve axons and therefore may not alter axonal excitability properties (Sindrup and Jensen, 1999).

From a clinical perspective, the present study has demonstrated that excitability techniques have the potential to identify DPN patients in whom alterations in Na^+ channel properties may be the dominant physiological abnormality. The study further demonstrates that these changes have a very clear clinical correlate, in that they are associated with greater impairments in QoL scores. At present, neuropathic pain treatments are administered largely on a 'trial and error' basis, reflecting the

contribution of multiple discrete mechanisms in the generation of diabetic neuropathic symptoms (Sindrup and Jensen, 1999, Yarnitsky *et al.*, 2012).

Current neuropathic treatments for DPN include anticonvulsant medications, tricyclic antidepressants and selective serotonin reuptake inhibitors, all of which block axonal Na^+ channels (Lenkey *et al.*, 2010). In contrast, GABA-mimetic agents, such as gabapentin and pregabalin, have clearly demonstrated benefits in the treatment of DPN-related symptoms (Rosenstock *et al.*, 2004) and yet have no specific effects on Na^+ channel properties (Lenkey *et al.*, 2010). These medications are thought to improve DPN symptoms through effects on the CNS, including enhancement of inhibitory input of GABA-mediated pathways and antagonism of excitatory NMDA receptors, thereby reducing central sensitization (Rose and Kam, 2002). Such medications may be less efficacious in patients in whom altered Na^+ conductances are a primary mechanism for neuropathic symptom generation. Importantly, the present study suggests that axonal excitability techniques, applied in the clinical setting, may have a role in determining the differential mechanisms underlying neuropathic symptom generation in DPN. Further to this, the study provides a basis for future work investigating whether excitability measures may play a role in clinical decision making pertaining to the commencement and monitoring of neuropathic pain treatment in patients with type 2 diabetes.

Chapter 2 – Mechanisms of axonal dysfunction in type 1 diabetes mellitus

SUMMARY

In the previous chapter, axonal dysfunction was characterized in type 2 diabetic patients. Specifically, neurophysiological parameters suggested ion channel dysfunction. In the present study, patients with type 1 diabetes were assessed in order to provide insights into the mechanisms of axonal dysfunction in this cohort.

Large cohort studies such as the DCCT and EDIC studies demonstrated that glycaemic control alone is inadequate for curtailing DPN in patients with type 1 diabetes. Animal models of type 1 diabetes suggest that functional and structural changes, specifically axo-glial dysjunction and C-peptide deficiency, may contribute to neuropathy development. The present study sought to examine and characterize axonal function in type 1 diabetic patients in the absence of clinical neuropathy. Thirty type 1 diabetic patients subsequently underwent sensory and motor nerve excitability studies to examine axonal function. Compared to controls, type 1 diabetic patients demonstrated significant axonal excitability abnormalities in both sensory and motor nerves, indicating axonal membrane depolarization. Mathematical modelling results demonstrated that these changes were due to reduced nodal Na^+ conductance, nodal/paranodal K^+ conductances and Na^+/K^+ pump dysfunction. Changes in these conductances are consistent with axo-glial dysjunction as outlined in animal models of type 1 diabetes. These were found in the absence of DPN. Accordingly, this study has provided support for functional alterations in axons of type 1 diabetic patients early in the disease. These data indicate a window of opportunity for prophylactic intervention and provide a platform for investigating concomitant C-peptide infusion as a neuroprotective measure in type 1 diabetes.

INTRODUCTION

Type 1 diabetes is characterized by the development of long-term macro- and microvascular complications including DPN. The findings from the Diabetes Control and Complications Trial (DCCT) and the Epidemiology of Diabetes Interventions and Complications (EDIC) study, indicate that in type 1 diabetic patients who are prescribed strict glycaemic control, there remains a high frequency of neuropathy development at long-term follow-up (Pop-Busui *et al.*, 2010). This anomaly is thought to be due to insulinopenia and loss of C-peptide, a proinsulin peptide involved in regulating insulin activity and phosphorylation of the insulin receptor (Grunberger *et al.*, 2001, Li *et al.*, 2001, Sima *et al.*, 2008). Previous studies in animal models of type 1 diabetes have suggested that loss of C-peptide leads to structural and functional changes in the axon (Sima, 1980, Brismar *et al.*, 1987, Grunberger *et al.*, 2001, Sima *et al.*, 2001, Sima, 2003, Sima, 2003) which subsequently alter axonal excitability (Sima *et al.*, 1986, Cherian *et al.*, 1996).

Previous studies of axonal excitability in diabetic patients, including those in Chapter 1, have focussed mainly on type 2 diabetic patients and have demonstrated disparate changes in multiple conductances including Na^+ conductances (Kitano *et al.*, 2004), K^+ conductances (Misawa *et al.*, 2005) and energy-dependent Na^+/K^+ pump function (Kwai *et al.*, 2013). The study presented in this chapter was undertaken to investigate the basis for axonal dysfunction in type 1 diabetes. Studies of motor and sensory excitability were conducted in type 1 patients who were relatively early in their disease course. Mathematical modelling of sensory and motor excitability was also undertaken to characterize the biophysical basis of altered axonal function (Bostock *et al.*, 1991, Kiernan *et al.*, 2005, Howells *et al.*, 2012).

METHODS

Forty-eight type 1 diabetic patients from the Prince of Wales hospital were recruited. Clinical characteristics such as age, sex, duration of diabetes diagnosis, BMI and HbA1c% were acquired.

All patients underwent comprehensive neurological screening using the Total Neuropathy Score (TNS) (Cornblath *et al.*, 1999). Thirty patients (M 15:F15) without signs of neuropathy were subsequently recruited.

The full suite of axonal excitability studies were performed on all patients in median motor and sensory axons as per standard TRONDNF protocol (as per Methodology). These included stimulus response behaviour, strength-duration relationships, threshold electrotonus, current threshold relationships and the recovery cycle paradigms. Of note, 10 patients also underwent additional tests during threshold electrotonus utilizing ±20% conditioning pulses. These studies were used for mathematical modelling of the axon in attempt to safeguard against testing artefacts that could arise from a depolarizing notch in threshold electrotonus (Burke *et al.*, 2007). Age and sex matched normal controls were also recruited and tested for comparison (n=21, average age 26.90±0.95 yrs and M12:F9).

Then either Spearman rho or Pearson correlations were undertaken between clinical characteristics and motor and sensory axonal excitability parameters previously shown to be related were undertaken. Student's t tests or Mann Whitney U tests were used where appropriate to compare motor axonal excitability parameters between controls and patients and likewise between sensory axonal excitability findings. Statistical significance was considered when $P<0.05$.

Mathematical modelling

The effect of type 1 diabetes on human motor and sensory axons was examined using the 'Bostock' model of axonal excitability (Bostock *et al.*, 1991, Kiernan *et al.*, 2005, Howells *et al.*, 2012). This model focuses on axonal properties that govern the nodal excitability of human axons as measured using the threshold tracking technique. The model consists of two space clamped compartments, namely a node and an internode, which are connected by pathways through and under the myelin sheath (Barrett & Barrett, 1982; Bostock *et al.* 1991). Voltage-gated fast and slow K^+ conductances were modelled at both the node and the internode, while persistent and transient Na^+ conductances were only present on the nodal membrane. Capacitative, leak and pump currents across the axolemma were modelled separately for the node and internode.

The model was adjusted to fit the mean control data separately for motor and sensory axons before fitting to the corresponding diabetes mean data. The model fitting followed an iterative least squares approach where the overall discrepancy was assessed and minimised using the weighted sum of the squares of the error terms between the modelled and group data.

RESULTS

Patient clinical characteristics

Patient clinical characteristics are collated in Table 2. 1. The patients had been diagnosed for a minimum of 6 months prior to enrolment and testing with a mean duration of diabetes at 123.85±18.77 months. Patients had a mildly elevated average BMI (26.10±0.85 kg/m^2) and slightly elevated HbA1c% (7.82±0.76). Random blood glucose levels at the time of testing were also elevated (8.98±1.19 mmol/L). All patients had normal sural and tibial nerve amplitudes with mean values of 18.69±1.13 μV and 15.88±0.77mV respectively and no sensory or motor symptoms or signs of peripheral neuropathy.

Table 2. 1. Clinical characteristics: type 1 diabetic patients

Clinical Characteristics	*Patients* *n = 30*
M : F	15 : 15
Age (years)	28.13 ± 1.77
BMI (kg/m^2)	26.10 ± 0.85
CSII : MDII	12 : 18
Duration of diabetes diagnosis (months)	123.85 ± 18.77
HbA1c%	7.82 ± 0.76
Random blood glucose (mmol/L)	8.98 ± 1.19
Sural amplitude (μV)	18.69 ± 1.13
Tibial amplitude (mV)	15.88 ± 0.77

Median motor axonal excitability findings

Stimulus response behaviour was normal in the patients with type 1 diabetes as expected in a non-neuropathic cohort. Importantly however, the patients demonstrated prominent abnormalities across multiple parameters of the threshold electrotonus testing paradigm which assess the behaviour of nodal and internodal conductances (Table 2. 2 and Figure 2. 1). These included significant reductions in threshold change during hyperpolarizing threshold electrotonus at 90-100ms (patients -108.80±2.48, controls -120.50±4.07, P=0.012), 20-40ms (patients -84.07±1.27, controls -90.91±1.46, P=0.004) and hyperpolarizing slope at 101-140ms (patients 1.77±0.05, controls 1.94±0.05, P=0.014). In depolarizing threshold electrotonus, there were significant reductions in threshold change during the undershoot phase (patients -16.48±0.58, controls-19.19±0.77, P=0.006), accommodation at 40ms (patients 20.84±1.23, controls 23.77±0.80, P=0.008) and accommodation during the S2 phase (patients 19.80±0.65, controls 23.55±0.70, P=0.007).

Abnormalities were also demonstrated in recovery cycle measures which explore changes in Na^+ and K^+ channel function after a single supramaximal impulse (Table 2. 2 and Figure 2. 1). There was a marked reduction in threshold change of subexcitability in the patient cohort (patients 10.44±0.60, controls 15.42±1.18, P<0.001). This was accompanied by a trend towards a reduced threshold change in superexcitability at 5ms (patients -22.48±1.21, controls -25.90±1.46, P=0.07). Measures of refractoriness did not significantly differ between controls and the patients with type 1 diabetes.

Median sensory axonal excitability

Sensory recordings demonstrated similar reductions in threshold change during threshold electrotonus and recovery cycle paradigms (Table 2. 3 and Figure 2. 1). Specifically, threshold change in depolarizing threshold electrotonus at 90-100ms (patients 45.77±0.67, controls 45.75±0.67, P=0.02), depolarizing undershoot (patients -18.4±0.66, controls -19.7±0.79, P=0.026), and during the S2 phase of accommodation (patients 15.97±0.53, controls 13.86±0.63, P=0.013) were significantly reduced. In hyperpolarizing threshold electrotonus, threshold change at TEh(10-20ms) (patients -75.45±0.82, controls -78.43±1.02, P=0.024) was also significantly reduced in the patients with type 1 diabetes (P<0.05). Hyperpolarizing I/V slope was also altered (patients 0.36 ± 0.01, controls 0.32 ± 0.01, P<0.04). As was noted in motor recordings, subexcitability demonstrated a prominent reduction in the patients with type 1 diabetes compared to control data (patients 7.87±0.43, controls 10.38±0.74, P<0.001).

Clinical characteristics such as glycaemic control and disease duration were correlated with excitability markers to establish a relationship between clinically relevant information and axonal function. There were no significant correlations between threshold electrotonus or recovery cycle parameters and markers of glycaemic control or disease duration.

Table 2. 2. Median motor excitability comparison: type 1 diabetic patients vs controls

Parameters (% change)	*Motor excitability studies*		
	Patients	*controls*	*P value*
Superexcitability at 5ms	-22.48 ± 1.21	-25.90 ± 1.46	0.072
Subexcitability	10.44 ± 0.60	15.42 ± 1.18	0.0002
TEh(90-100ms)	-108.80 ± 2.48	-120.50 ± 4.07	0.012
TEd(10-20ms)	65.16 ± 0.75	67.24 ± 0.91	0.078
TEh(10-20ms)	-69.72 ± 0.80	-72.70 ± 0.86	0.016
TEd(undershoot)	-16.48 ± 0.58	-19.19 ± 0.77	0.006
TEd(peak)	65.79 ± 0.78	68.02 ± 0.95	0.070
TEh(20-40ms)	-84.07 ± 1.27	-90.91 ± 1.46	0.004
TEh slope (101-140ms)	1.77 ± 0.05	1.94 ± 0.05	0.014
TEd(40 Accommodation)	20.84 ± 1.23	23.77 ± 0.80	0.008
S2 Accommodation	19.80 ± 0.65	23.55 ± 0.70	0.007

Overall, median motor axons in type 1 diabetic patients had less threshold change in response to stimuli during the recovery cycle and threshold electrotonus at multiple time points. Statistical significance was denoted when P value < 0.05.

Table 2. 3. Median sensory excitability comparison: type 1 diabetic patients vs controls

Parameters (% change)	*Sensory excitability studies*		
	Patients	*controls*	*P value*
Subexcitability	7.87 ± 0.42	10.38 ± 0.74	0.003
TEd(90-100ms)	45.77 ± 0.67	45.75 ± 0.67	0.028
TEh (10-20ms)	-75.45± 0.82	-78.43 ± 1.02	0.024
TEd(undershoot)	-18.40 ± 0.66	-19.70 ± 0.79	0.026
S2 Accommodation	15.97 ± 0.53	13.86 ± 0.63	0.013

Overall, median sensory axons in type 1 diabetic patients had lower threshold change in response to stimuli during the recovery cycle and threshold electrotonus at multiple time points. Values are given as percentage change in threshold (%). Statistical significance was denoted when *P* value < 0.05.

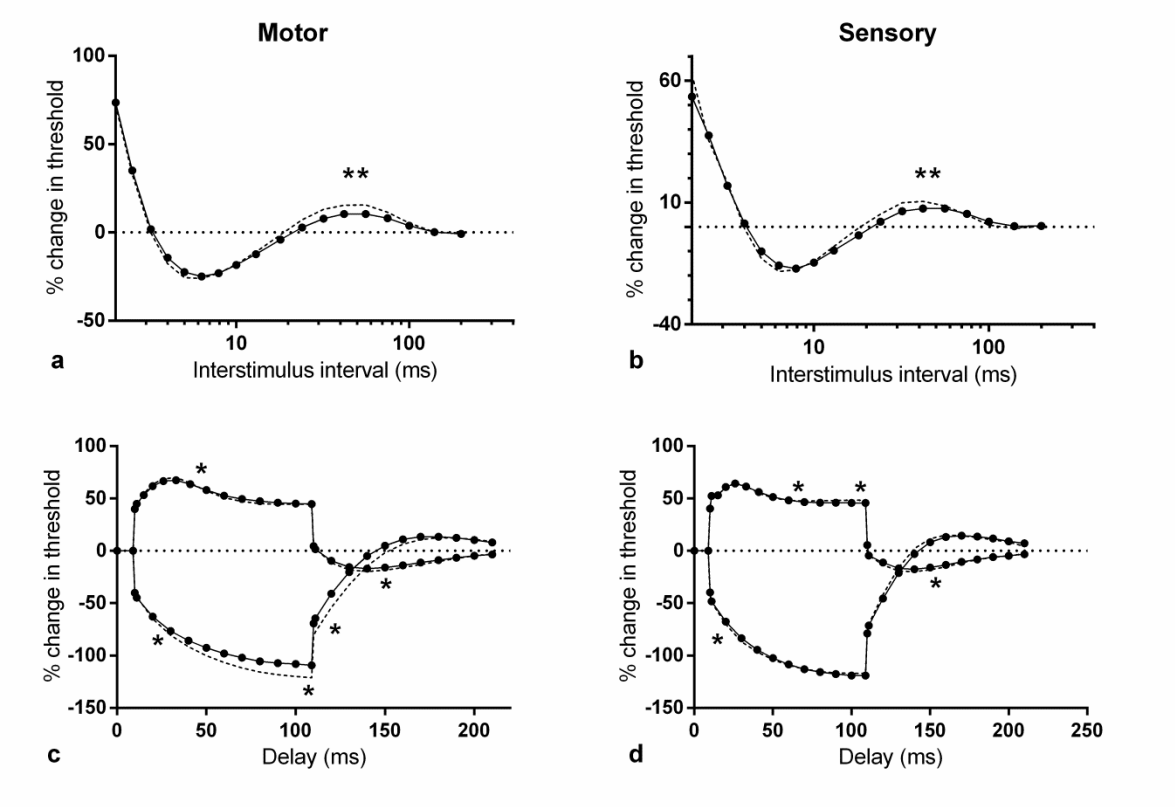

Figure 2. 1. Motor and sensory excitability recordings for recovery cycle and threshold electrotonus in patients vs controls

Group mean data for recovery cycle and threshold electrotonus paradigms are plotted above. Dotted lines indicate controls and solid black lines with circles represents patients with type 1 diabetes. Motor recovery cycle and threshold electrotonus (a and c) are on the left panel and corresponding sensory plots are on the right panel (b and d). Patient mean data is significantly reduced across multiple parameters. * indicate where $P<0.05$ and ** where $P<0.01$.

Mathematical modelling of changes in axonal function

Application of mathematical modelling found that simple alterations in membrane potential or in individual channel conductances alone could not account for the differences in the axonal excitability findings in the patients with type 1 diabetes. However, a combination of currents on either side of the paranode, the domain of the axon demarcated by the junction between Schwann cells and the axolemma (axo-glial junction), reduced the discrepancy between modelled normal data and the motor and sensory axons of the patients with type 1 diabetes by 59 and 90%, respectively (Figure 2. 2). The modelled parameters are shown in Table 2. 4, with the key differences involving a reduction in nodal Na^+, slow K^+ currents, and internodal fast K^+ currents. Additionally, a 5 pA reduction in Na^+/K^+ pump currents was also noted in the modelled sensory data from the patients, an important finding given that previous studies have found this energy dependent pump to be dysfunctional in DPN (Greene, 1986, Edwards *et al.*, 2008). The modelling suggests that the combined effect of these changes is a depolarization of the nodal resting membrane potential by 1.1 and 3 mV for motor and sensory axons respectively in the patients.

Table 2. 4. Modelled excitability parameter changes in type 1 diabetic patients

Parameter	*Motor*	*Sensory*
Max. permeability of Na^+ channels at the node ($cm^3 \times s^{-1} \times 10^{-9}$)	3.65 (4.5)	3.4 (4.35)
Max. conductance of slow K^+ channels at the node (nS)	40.8 (52.3)	22.4 (29.1)
Max. conductance of slow K^+ channels at the internode (nS)	0.36 (0.36)	0.44(1.74)
Max. conductance of fast K^+ channels at the node (nS)	28.5 (25)	27.5 (26)
Max. conductance of fast K^+ channels at the internode (nS)	37.5 (50)	62.5 (188)
Barrett-Barrett conductance (nS)	35.9 (35.9)	36 (32.2)
Reduction in the Na^+/K^+-ATPase pump current (pA)	0	5
Resting membrane potential (mV)	-81.1 (-82.2)	-78.8 (-81.8)

The key changes applied in mathematical model that produce the pattern of change seen in the patients when compared to controls are listed above. Patient values are listed in the motor and sensory columns and modelled normal control data is bracketed below each parameter. The discrepancy between patient and control data was explicable by reductions Na^+ and slow and fast K^+ conductances at the node, reduced slow K^+ at the internodal region, reduced Barrett-Barrett conductances, reduced Na^+/K^+ pump currents and membrane depolarization

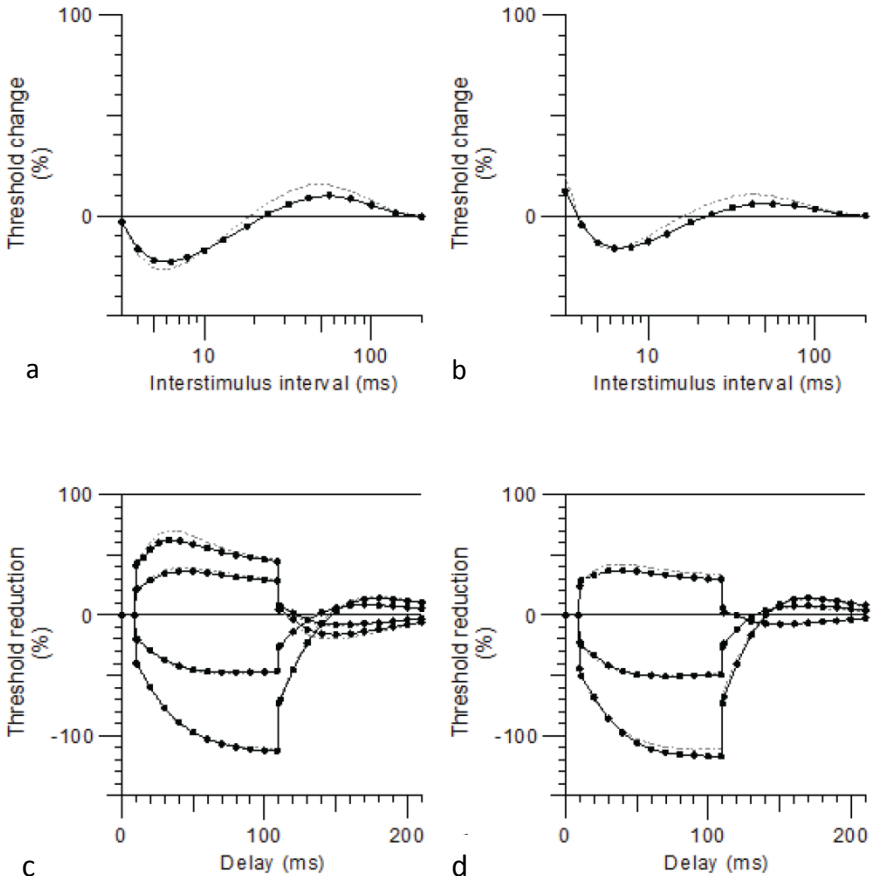

Figure 2. 2. Mathematical modelling results for motor and sensory recovery cycle and threshold electrotonus in patients vs controls

Motor recovery cycle and threshold electrotonus (a and c) are on the left panel and corresponding sensory plots are on the right panel (b and d). Dashed lines represent modelled normal control data and block line represents modelled patient data. Note that 40% depolarization is not modelled for sensory axons, as this level of depolarization is usually not subthreshold. The appearance of the modelled data, which is qualitatively similar to what was seen in the patients, was achieved by modelling current changes that would occur with axo-glial dysjunction: reduced Na^+ conductances at the node, reduced slow and fast K^+ conductances at the node and paranode (respectively) and reduced Na^+/K^+ pump activity.

DISCUSSION

Axonal excitability measures represent surrogate markers of alteration in voltage-gated conductances that are involved in the process of impulse conduction in peripheral nerves. The present study has uncovered prominent changes in axonal excitability in patients with type 1 diabetes. These findings have been demonstrated in a cohort of patients who have no clinical features of neuropathy. The potential cause for these changes is therefore unlikely to be due to axonal loss, which is more typical of established neuropathy: the changes are more likely to represent an early functional change in the nerves of patients with type 1 diabetes.

The excitability changes were noted in threshold electrotonus and recovery cycle parameters and included reductions in both depolarizing and hyperpolarizing threshold electrotonus, which were accompanied by prominent reductions in subexcitability and in superexcitability in motor recordings. Based on previous studies, the changes in threshold electrotonus may be interpreted as abnormalities of outwardly rectifying K^+ channels (Baker *et al.*, 1987) while the changes in superexcitability and subexcitability would be consistent with altered Na^+ channel function (Kiernan *et al.*, 2005). Persistent Na^+ conductances, indicated by SDTC, were not prolonged in patients compared to controls. This is in contrast to the previous chapter in this thesis which indicated that SDTC progressively lengthened with increasing neuropathy severity and correlated with neuropathy specific quality of life driven by ectopic symptom scores (Kwai et al., 2013). The present study however, was undertaken in a cohort of type 1 diabetic patients who were non-neuropathic. It is therefore possible that prolongation of SDTC in diabetic patients only occurs in a subset of patients, possibly those in whom excitability changes have

led to the development of ectopic sensory symptoms. Regardless of the above, interpretation of excitability abnormalities in the clinical context can be difficult as changes even in a single conductance can lead to compensatory changes in other voltage-dependent conductances that may confound interpretation of excitability parameters.

The recent development of a validated mathematical model for human motor and sensory axons has assisted with interpretation of excitability data, and has been successfully used in the assessment of peripheral nerve function in other metabolic conditions including porphyria and chronic kidney disease (Lin *et al.*, 2008, Arnold *et al.*, 2014). Mathematical modelling of the sensory and motor recordings in the present study identified a number of important biophysical changes. The most prominent of these was a marked reduction in nodal Na^+ currents in the axons of patients with type 1 diabetes. These changes were also accompanied by a reduction in nodal slow K^+ and juxtaparanodal fast K^+ currents and altered Na^+/K^+ pump function. Together these changes in K^+ channels and Na^+/K^+ pump dysfunction may underlie depolarization of the resting membrane potential.

The most prominent change uncovered by modelling in the present study was a marked reduction in nodal Na^+ currents in both sensory and motor recordings. Possible causes for the alterations in Na^+ conductances include changes in nodal Na^+ channel density or reduction in the electrochemical gradient for Na^+. Molecular studies have recently identified that alterations in insulin signalling and hyperglycaemia-induced activation of the polyol pathway may lead to biophysical abnormalities of axons in type 1 diabetes (Sima and Zhang, 2014). Insulin has been shown to have potent neurotrophic properties and insulin receptors are concentrated

in nodal and paranodal regions of myelinated axons (Sugimoto *et al.*, 2000, Sima and Zhang, 2014). Insulin signalling plays an important role in ensuring normal function of the energy-dependent axonal Na^+/K^+ pump and studies have shown that impaired insulin signalling is involved in Na^+/K^+ pump dysfunction through a P13-kinase mechanism (Greene *et al.*, 1992, Sima, 2003).

Polyol pathway activation is an additional contributing factor that may exacerbate Na^+/K^+ pump dysfunction by increasing intracellular sorbitol, subsequently depleting myo-inositol that further limits protein kinase C activity (Greene *et al.*, 1987). Protein kinase C activity is crucially involved in Na^+/K^+ pump function and thus a reduction in its activity reduces pump activity. A consequence of Na^+/K^+ pump dysfunction is intracellular retention of Na^+, which causes a change in the electrochemical gradient for Na^+ entry. This abnormality also induces osmotic changes in the axon that lead to nodal and paranodal swelling with spatial deformation of the paranodal apparatus and loss of junctional complexes between myelin loops and the paranodal axolemma, a phenomenon known as 'axo-glial' dysjunction (Sima *et al.*, 1986) . These structural changes have been shown to induce alterations in ion channel localisation, including lateral displacement of Na^+ channels away from the node and into the internodal region (Brismar *et al.*, 1987, Cherian *et al.*, 1996, Dupree *et al.*, 1999, Rosenbluth, 2009). Recent studies have also indicated that insulin signalling itself plays an important role ion channel localisation by modifying the biological activity of molecules such as ankyrin, contactin and caspr which anchor voltage-gated Na^+ and K^+ ion channels into specialised regions of the axolemma (Dupree *et al.*, 1999). Reduced expression of these molecules may lead to movement of these channels away from nodal and paranodal regions and these changes may explain the reductions in nodal and paranodal K^+ conductances noted in

the present study whilst also contributing to the reduced inward Na^+ current (Sima *et al.*, 2004).

Chapter 3 – Neuroprotective potential of insulin treatment regimens

SUMMARY

In Chapter 2, axonal dysfunction occurred in type 1 diabetic patients in the absence of clinical signs and symptoms of neuropathy, suggestive of a window of opportunity for neuroprotective treatment in type 1 diabetic patients. Although strict glycaemic control may reduce the risk of developing DPN, the neurological benefits of different insulin regimens remain relatively unknown.

In the present study, 51 consecutive patients with type 1 diabetes underwent clinical neurological assessment. Subsequently, 41 non-neuropathic patients, 24 of whom were receiving multiple daily insulin injections (MDII), and 17 receiving continuous subcutaneous insulin infusion (CSII), underwent motor axonal excitability testing. Treatment groups were matched for glycaemic control, BMI, disease duration and gender and neurophysiology parameters were compared between treatment groups and those taken from age and sex matched normal controls.

Prominent differences in axonal function were noted between MDII- and CSII-treated patients. Specifically, MDII-treated patients manifested prominent abnormalities when compared to normal controls in threshold electrotonus properties including TEd(10-20ms),undershoot and TEh(90-100ms) ($P<0.05$). Additionally, recovery cycle parameters superexcitability and subexcitability were also abnormal ($P<0.05$). In contrast, axonal function in CSII-treated patients was within normal limits when compared to age matched controls. These differences between the groups were noted in cross-sectional analysis and remained at longitudinal follow-up.

These findings indicate that axonal function in diabetes is maintained within normal limits in patients treated with CSII and not MDII. This raises the possibility that CSII

therapy may have neuroprotective potential in patients with type 1 diabetes and indicate that this action may be independent of overall glycaemia (HbA1c%).

INTRODUCTION

When compared to type 2 diabetes, patients with type 1 diabetes may develop a more severe phenotype, possibility mediated by insulinopenia and C-peptide deficiency which are required for optimal axonal metabolism and function (Sima *et al.*, 2004, Sima and Kamiya, 2006). Recent studies utilizing axonal excitability techniques have suggested more prominent abnormalities in axonal function in patients with type 1 diabetes mellitus compared to patients with type 2 in the absence of neuropathy (Arnold *et al.*, 2013).

Studies in diabetic patients have demonstrated prominent changes in axonal ion channel function in both type 1 and type 2 diabetic patients suggesting altered axonal Na^+ conductances and axonal Na^+/K^+ pump dysfunction (Kuwabara *et al.*, 2002, Kuwabara, 2003, Misawa *et al.*, 2005, Misawa *et al.*, 2006). The findings of this thesis and other studies have found that these abnormalities are present in patients with established DPN (Krishnan and Kiernan, 2005), correlate strongly with neuropathy-related QoL (Kwai *et al.*, 2013) and are consistent with animal models of DPN (Craner *et al.*, 2002, Hong *et al.*, 2004, Hong and Wiley, 2006). Furthermore, these changes are also present prior to the development of clinical neuropathy (Sung *et al.*, 2012, Arnold *et al.*, 2013).

Treatment of DPN consists of alleviation of neuropathic symptoms and optimisation of glycaemic control (Brussee *et al.*, 2004).The DCCT and EDIC trial found that strict glycaemic control through intensive insulin therapy achieved by multiple daily insulin injections (MDII) or continuous subcutaneous insulin infusion (CSII) was associated with reduced prevalence and delayed progression of DPN in type 1 diabetes (Pop-Busui *et al.*, 2010). The benefits of CSII over MDII include reduction

in the number of severe hypoglycaemic events, lowering of glucose exposure and improved treatment satisfaction for diabetic patients (Bode *et al.*, 1996, Hirsch *et al.*, 2005, Hoogma *et al.*, 2006, Pickup and Renard, 2008, Yeh *et al.*, 2012). However, the potential differences between the two regimens in terms of their effect on peripheral axonal function have not been systematically investigated.

The major aim of this study was to utilize axonal excitability techniques to investigate whether there were differences in axonal function of patients with type 1 diabetes treated with either CSII or MDII therapy. A secondary aim was to establish whether these changes may be detected prior to the onset of clinical symptoms or nerve conduction evidence of neuropathy. Lastly, we wanted to investigate if these abnormalities were present at longitudinal follow-up in a subgroup of tested patients.

METHODS

A total of 55 patients with type 1 diabetes mellitus were consecutively recruited and assessed at the Prince of Wales Hospital in Sydney. All patients had been on their respective insulin treatment type for a minimum of 12 months prior to testing. Clinical characteristics were obtained, including BMI, duration of diabetes diagnosis, random blood glucose by finger-stick at the time of testing and HbA1c% at the time of testing and for two year prior to assessment. All patients underwent a comprehensive neurological examination by an examiner blinded to treatment to determine presence of DPN.

Patients without clinical signs of neuropathy (n=41) subsequently underwent assessment of motor axonal excitability of the median nerve as per Methodology. Specifically, threshold electrotonus parameters TEd(10-20ms), undershoot, accommodation and TEh(90-100ms) and recovery cycle parameters super and subexcitability. In a subgroup of 18 patients, excitability assessments were performed at longitudinal follow-up (6-12 month follow-up).

Patient clinical and axonal excitability assessments results were then grouped and compared according to insulin regimen: CSII (n=17) and MDII (n=24). Specifically, MDII consisted of at least three injections per day of rapid acting 9 Aspart, Lispro and either glargine and detemir. CSII treatment involved a basal infusion of insulin with a large bolus infusion at meals. Twenty age matched and gender matched normal controls were also recruited and underwent axonal excitability assessments for comparison to patient results.

Statistical Analysis

Statistical analysis was performed using SPSS statistics software v. 20 (IBM, Chicago, IL). Assessments of normality were first undertaken on patient group data using the Shapiro Wilk test. To determine if axonal dysfunction occurs in non-neuropathic patients with type 1 diabetes, axonal excitability results from all non-neuropathic patients (n=41) were compared to controls (n=20) using Student's t tests or Mann Whitney U tests where appropriate. To establish if there were differences in axonal function between patients treated with MDII and CSII, axonal excitability parameters from the MDII-treated and CSII-treated groups were compared to each other and normal controls. To investigate potential relationships between clinical characteristics, NCS and neurophysiological measures, Spearman Rho or Pearsons correlations were undertaken where appropriate. Mean values where provided are expressed as mean $\pm$ SEM. Findings were considered statistically significant when $P<0.05$.

RESULTS

The clinical and demographic characteristics for the patients (n=41) are shown in Table 3. 1. Seventeen patients were receiving continuous insulin infusion (CSII) therapy and 24 were receiving multiple daily insulin injections (MDII). Patients receiving CSII had all previously been treated with a standard MDII regimen at the time of initial diagnosis and subsequently switched to CSII therapy. The average duration of CSII treatment was 5.38 ±1.43 years. No correlations were found between neurophysiology parameters and duration of CSII therapy. There were also no significant differences in glycaemic control, duration of diabetes, age, gender, BMI, total insulin requirement or sensory or motor amplitudes between the two treatment groups, although tibial latencies were faster in the CSII treated group. Additionally, 5 patients receiving MDII treatment presented with ophthalmic evidence of mild but stable retinopathy compared to only 1 patient treated with CSII. No signs of nephropathy were noted in the patient cohort as established by an estimated glomerular filtration rate > 90ml/kg/m^2 and no evidence of microalbuminuria. Clinically notable hypoglycaemic events were reported by 3 patients (2 CSII: 1 MDII) within the week prior to assessment.

Table 3. 1. Clinical characteristics: comparison between MDII and CSII

	Treatment Type			
	All patients	*MDII*	*CSII*	*p*
	n=41	*n=24*	*n=17*	*value*
M:F	23:18	14:10	9:8	
Age (yrs)	27.66 ± 1.37	27.50 ± 1.40	27.88 ± 2.71	*0.33*
DOD	119.69 ± 15.68	112.86 ± 19.32	130.43 ± 25.25	*0.57*
HbA1c %	7.76 ± 0.21	7.74 ± 0.27	7.79 ± 0.34	*0.92*
(2 year average)	8.30 ± 0.28	8.51 ± 0.35	7.92 ± 0.46	*(0.21)*
RBG (mmol/L)	10.13 ± 0.97	10.64 ± 1.40	9.35 ± 1.22	*0.48*
BMI (kg/m ^{2})	25.29 ± 0.69	25.23 ± 0.77	26.09 ± 1.28	*0.73*
Average daily insulin (units)	51.97 ± 3.94	54.45 ± 28.45	48.32 ± 15.63	*0.74*
Sural amplitude (μv)	18.44 ± 0.97	19.60±1.37	17.11±0.32	*0.54*
Sural velocity (m/s)	43.72 ± 7.30	41.51±1.45	46.75±1.48	*0.07*
Tibial amplitude (mV)	15.68 ± 0.83	15.32±1.23	16.16±1.10	*0.44*
Tibial latency (ms)*	3.35 ± 0.06	3.49±0.05	3.16±0.07	*0.02*

Values are given as mean±SEM. Continuous subcutaneous insulin infusion (CSII), multiple daily insulin injections (MDII), duration of diabetes (DOD), random blood glucose (RBG). CSII vs MDII: * $P<0.05$.

When compared to age and gender matched normal controls (n=20), the patients as a group demonstrated altered axonal nerve excitability parameters (Table 3. 2). Of note, there was a reduced percentage change in hyperpolarizing threshold electrotonus at 90-100 ms (patients -110.40±2.41, controls -121.20±3.89, $P<0.05$) and TEd undershoot (patients -16.46±0.45, controls -18.95±0.74, $P<0.01$). Changes were also noted in recovery cycle parameters, specifically superexcitability (%) which was reduced in patients (-22.18±0.01) compared to controls (-26.47±1.20, $P<0.05$) and reduced subexcitability (patients 11.15±0.53, controls 15.02±0.03, $P<0.01$). The pattern of change in excitability parameters is consistent with axonal membrane depolarization (Kiernan and Bostock, 2000) and agrees with previous findings of this thesis in patients with type 1 diabetes and published in part by Arnold et al. (2013) (Arnold *et al.*, 2013).

Table 3. 2. Median motor excitability comparison: MDII vs CSII vs controls

Excitability parameters	All patients n=41	MDII n=24	CSII n=17	Controls n=20
1. Initial test				
Threshold Electrotonus				
TEd(10-20ms)	65.22±0.70*	63.16±0.78**†	68.13±3.89	67.80±1.08
TEd(accommo40ms)	20.73±0.53*	19.58±0.53**†	22.35±0.92	22.81±0.83
TEd(undershoot)	-16.46±0.45**	-15.74±0.53**	-17.47±0.71	-18.98±0.74
TEh(90-100ms)	-110.40±2.41*	-103.80±2.47**††	-119.60±3.67	-121.20±3.89
Recovery Cycle				
Superexcitability	-22.18±0.01*	-22.17±0.84**†	-24.12±1.08	-26.47±1.20
Subexcitability	11.15±0.53**	10.14±0.44**†	12.51±1.03	15.02±0.03
2. Follow-up	n=18	n=12	n=6	n=20
Threshold Electrotonus				
TEd(10-20ms)	64.76±1.09	63.21±0.85*†	68.70±2.11	67.80±1.08
TEd(undershoot)	-16.01±0.70*	-16.27±0.64*	-18.98±0.74	-18.98±0.74
TEh(90-100ms)	-111.00±0.01	-102.06±4.60*†	-121.02±6.62	-121.20±3.89
Recovery Cycle				
Superexcitability	-23.82±1.02*	-22.98±1.35**	-26.47±1.20	-26.47±1.20
Subexcitability	-10.54±0.74*	9.76±0.79*	12.00±1.45	15.02±0.03

Table 3.2. Nerve Excitability values for the treatment groups and controls at initial assessment (1) and 6-12 month follow-up (2). Values are expressed as percentage change in threshold (mean±SEM). Continuous subcutaneous insulin infusion (CSII) and multiple daily

insulin injections (MDII). All axonal excitability parameters were normally distributed. The MDII-treated group show significant abnormality across numerous parameters when compared to the CSII group and controls. These changes are indicative of axonal membrane depolarization. Compared to controls: * $P<0.05$ and ** $P<0.01$. Compared to CSII: † $P<0.05$ and †† $P<0.01$.

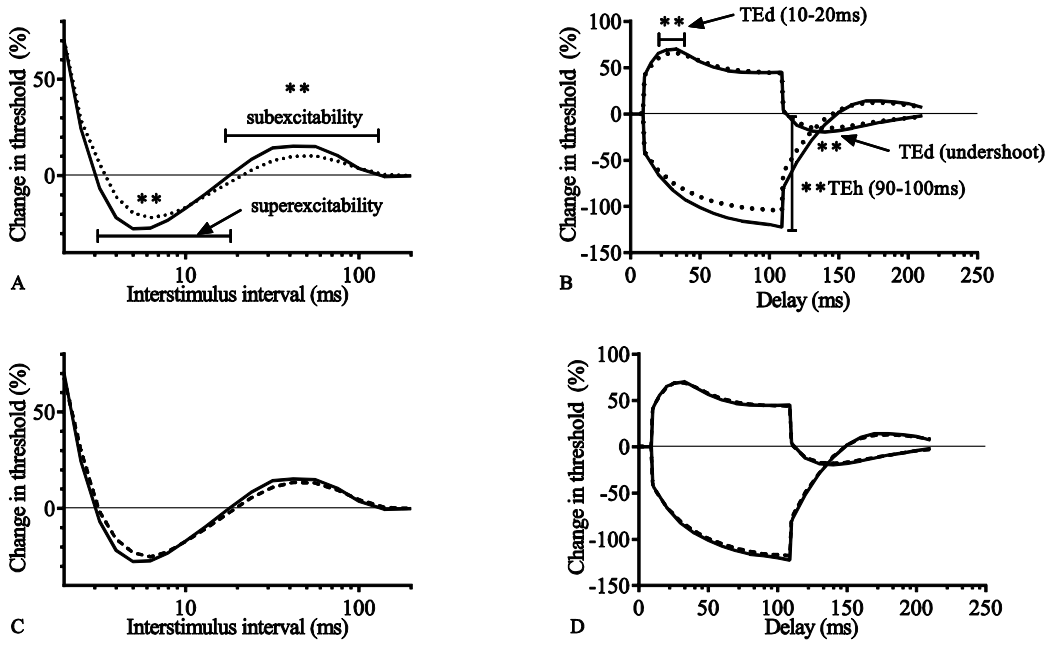

Figure 3. 1. Motor excitability plots for recovery cycle and threshold electrotonus: MDII vs CSII

Block lines =mean control data, large dashed line = mean data for CSII-treated patients and dotted line= mean data for MDII-treated patients. There is a flattened appearance of the MDII-treated group mean plots in recovery cycle (a) and threshold electrotonus (b) compared to controls. Conversely, CSII-group mean plots for recovery cycle (c) and threshold electrotonus (d) were similar to normal control plots. Compared to controls:* $P<0.05$, ** $P<0.01$.

When analysed according to treatment type, prominent abnormalities of axonal function were noted in the MDII group compared to both CSII-treated patients and normal controls (Table 3. 2 and Figure 3. 1). Specifically, in the MDII group reduced threshold change (%) and concurrent flattening of mean plots was noted in the recovery cycle parameters superexcitability (MDII -22.17±0.84, controls -26.47±1.20, $P<0.01$) and subexcitability (MDII 10.14±0.44, controls 15.02±0.03, $P<0.01$). Likewise, reduced threshold change in depolarizing (TEd) and hyperpolarizing (TEh) threshold electrotonus was found at multiple time points: TEd(10-20ms) (MDII 63.16±0.78, controls 67.80±1.08, $P<0.01$), TEd undershoot (MDII -15.74±0.53, controls -18.98±0.74, $P<0.01$), and TEh(90-100ms)(MDII -103.80±2.47, controls -121.20±3.89, $P<0.01$) (Figure 3. 1). Additionally, compared to the CSII –treated group, MDII-treated patients had significantly reduced threshold change in TEd(10-20)(CSII 68.13±3.89, $P<0.05$), TEd(acccommo40ms)(CSII 22.35±0.92, $P<0.05$), TEh(90-100ms) (CSII-119.60±3.67 , $P<0.01$), superexcitability (CSII -24.12±1.08, $P<0.05$) and subexcitability (CSII 12.51±1.03, $P<0.05$)(Table 3. 2).

In contrast to the MDII-treated group, the CSII-treated group demonstrated no significant change in excitability parameters compared to controls (Table 3. 2). Specifically, there were no significant changes in threshold electrotonus parameters or in measures of the recovery cycle, with mean data plots for CSII-treated patients showing relative normality compared to control plots (Figure 3. 1).

Longitudinal assessment of axonal function

Longitudinal studies were undertaken at 6-12 months on a subgroup of the patient cohort (n=18). Over this period of time, there had been no significant change in clinical features or measures of glycaemic control in either group. The previously demonstrated abnormalities in excitability parameters persisted in the MDII group (n=12) (Table 3. 2). Significant abnormalities were again noted in threshold electrotonus and recovery cycle parameters. In threshold electrotonus, TEd(10-20ms), TEd(undershoot) and TEh(90-100ms)(P<0.05) (Figure 3. 2a and b) were significantly different in MDII-treated patients compared to controls with TEd(10-20ms) also showing reductions compared to the CSII subgroup at follow-up (CSII 68.70$\pm$2.11, P<0.05). Likewise, recovery cycle parameters superexcitability (MDII -22.98$\pm$1.35, P<0.01) and subexcitability (MDII 9.76$\pm$0.79, P<0.05) (Figure 3. 2c) were again significantly reduced. In contrast to the MDII group, CSII-treated patients continued to maintain normal axonal function.

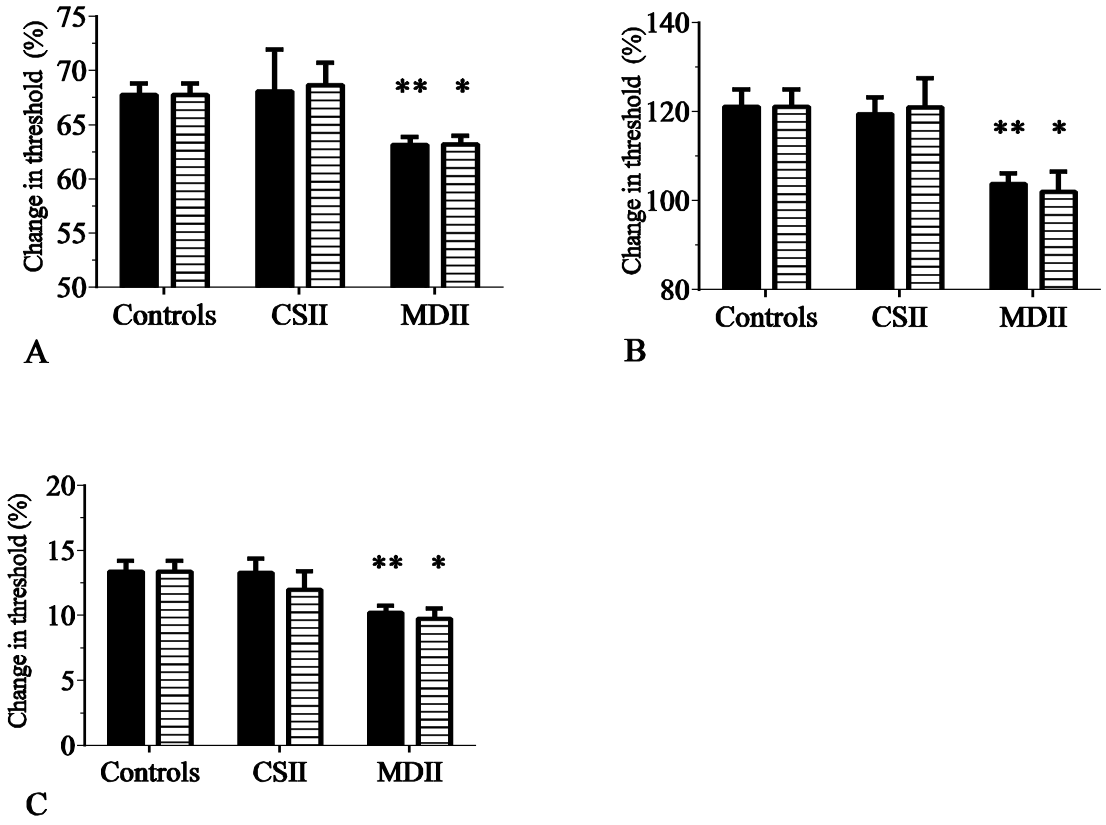

Figure 3. 2. Motor excitability comparison: controls vs treatment groups at follow-up

Block columns represent mean values ± SEM at baseline and striped columns represent mean values ± SEM at 6-12 month follow-up. Mean values ±SEM are plotted for TEd(10-20ms) (a), TEh(90-100ms) (b) and subexcitability (c) and are expressed as percentage change in threshold. Significantly reduced % change in threshold was noted in the MDII group compared to controls and the CSII-treated groups across multiple parameters at initial and follow-up assessments. Compared to controls: * $P<0.05$ and ** $P<0.01$.

DISCUSSION

The present study has demonstrated that patients with type 1 diabetes that are treated with MDII have abnormal axonal function when compared to age matched controls and CSII-treated patients. These changes were noted in cross-sectional studies and remained at longitudinal follow-up. In addition, faster lower limb motor nerve conduction was noted in CSII-treated patients, demonstrating that CSII is associated with improved axonal function in those regions that are most susceptible to the development of neuropathy, namely the distal lower limbs. All patients had been receiving their respective treatment type for at least 12 months prior to enrolment and the choice of treatment was based largely on patient preference or, in some cases, advised by the treating physician due to the occurrence of nocturnal hypoglycaemic episodes. However, although patients were assessed by an investigator blinded to treatment, the lack of random assignment to treatment remains a potential limitation of the study.

In contrast to CSII-treated patients, the present study demonstrated that MDII-treated patients had significant abnormalities of excitability. Prominent changes were noted across a number of threshold electrotonus and recovery cycle parameters in a pattern that is consistent with depolarization of the axonal membrane potential (Kiernan and Bostock, 2000). The potential basis for membrane depolarization may relate to dysfunction of the energy-dependent axonal Na^+/K^+ pump. The Na^+/K^+ pump is responsible for maintaining a normal axonal electrochemical gradient, and reduced activity of the Na^+/K^+ pump has been demonstrated in nerve preparations from type 1 and type 2 diabetic patients (Scarpini *et al.*, 1993). Studies in animal models of type 1 diabetes have also suggested that Na^+/K^+ pump dysfunction may occur due to

upregulated polyol pathway activity in the presence of hyperglycaemia, oxidative stress and C-peptide deficiency (Greene and Lattimer, 1984, Sima *et al.*, 2000, Sima and Kamiya, 2006). In these studies changes in excitability were limited to hyperpolarizing threshold electrotonus. The changes in the present study however encompass a range of threshold electrotonus and recovery cycle parameters, arguing against a selective alteration in a single conductance in motor axons.

As CSII is associated with improved glycaemic control (Pickup, 2012, Yeh *et al.*, 2012) it could be argued that the differences in axonal function were related to better glycaemic control or differences in the total amounts of insulin administered in the two groups. However, the present study failed to establish differences in glycaemic control between the two treatment groups or to find correlations between HbA1c% and nerve excitability parameters. Previous work has provided evidence of changes in nerve conduction velocity and intraepidermal nerve fibre with alterations in insulin dosing, independent of changes in glucose levels (Brussee *et al.*, 2004, Toth *et al.*, 2006, Sugimoto *et al.*, 2013). However there were no significant differences between the two treatment groups in terms of total insulin requirement in the present study. Additionally, no correlations were found between duration of insulin treatment through CSII and neurophysiological parameters.

An alternative explanation for the differences between treatment groups may relate to differences in blood glucose variability, which has been linked to overall morbidity (Wintergerst *et al.*, 2006), microvascular dysfunction (Kilpatrick *et al.*, 2006) and oxidative stress in diabetic subjects (Quagliaro *et al.*, 2003, Monnier *et al.*, 2006). It is well established that oxidative stress is well linked to complications in diabetes (Vincent *et al.*, 2004) in particular endothelial dysfunction (Su *et al.*, 2008) and

subsequently compromised microvasculature function (Giannini and Dyck, 1994). Previous studies have found less glucose variability in patients treated with CSII compared to MDII (Bruttomesso *et al.*, 2008). We speculate that the CSII-treated patients in the present study may have had a reduced variability in glucose profile than the MDII-treated patients, thus protecting CSII-treated patients from the consequences of microvascular dysfunction and oxidative stress. The present study did not quantify glucose variability however, further studies incorporating continuous glucose monitoring would be needed to assess the effects of glucose variability and direct insulin signalling on axonal function.

From a clinical perspective, the findings of the present have important implications. While the duration of follow-up in the present study was limited to 12 months in a subgroup of the initial cohort, longer periods of assessment in a larger cohort may help delineate whether the better axonal function in CSII-treated patients results in better clinical outcomes. The basis for this relates to the previously defined association between nerve ion channel dysfunction and axonal degeneration (Stys, 2005). Chronic changes in axonal ion channel function have been shown to trigger a cascade of events resulting in reverse operation of Na^+/Ca^+ exchanger, causing an excitotoxic Ca^+ influx and subsequent axonal degeneration (Lehning *et al.*, 1996, Stys, 2005). This study also suggests that nerve excitability techniques may be able to identify a window of opportunity for which therapeutic interventions targeting axonal ion channel function in patients may be useful. Although the present study established differences and magnitude of these differences in axonal function between CSII and MDII, further investigation to clarify the effects of insulin treatment regimens is required using larger randomised control trials and crossover studies.

In total, the findings of the present study suggest that non-neuropathic type 1 diabetic patients treated with CSII have normal axonal ion channel function compared to MDII-treated patients with similar clinical characteristics. The altered axonal function noted in MDII-treated patients was similar to that seen in patients with established DPN and is indicative of axonal membrane depolarization. The findings of this study are relevant clinically as they as they raise the possibility that CSII therapy may have neuroprotective potential in type 1diabetes. These data provide the basis for further investigation in a clinical trial setting of the differential effects of insulin treatment regimens on axonal function in type 1 diabetes.

Chapter 4 – Effect of glucose variability on axonal function in type1 diabetes mellitus

SUMMARY

In Chapter 3, CSII-treated type 1 diabetic patients were found to have relatively preserved axonal function compared to MDII-treated patients. However the mechanisms for this finding remain unclear. It is possible that differences are due to glycaemic variability which has been put forward as a benefit of CSII therapy. In this chapter, the role of glycaemic variability and acute glucose levels on motor and sensory axonal function in type 1 diabetic patients was explored utilizing continuous glucose monitoring and axonal excitability techniques at different blood glucose ranges.

Axonal excitability studies were undertaken in 12 type 1 diabetic patients. Median motor and sensory axonal excitability studies were obtained at high (>10mmol/L), medium (4-10mmol/L) and low (<4mmol/L) blood glucose ranges in all patients on a single day. Mean amplitude of glycaemic excursions (MAGE), a measure of glycaemic variability, was assessed in all patients over a 48-hour period using a subcutaneous glucose sensor. At all glucose ranges (high, medium and low), mean patient data showed reduced threshold change in threshold electrotonus, recovery cycle and current threshold relationships as noted in previous chapters. Mixed model analysis revealed no significant differences between high, medium and low range mean excitability data for either motor or sensory studies. Importantly, mean amplitude of glycaemic excursions (MAGE), a measure of glycaemic variability, was strongly correlated with motor excitability parameters including minimum I/V slope (r= 0.89), superexcitability (r=0.62), S2 Accommodation (r=-0.71) and TEd (40 Accommodation) (r=-0.70) (P<0.05). In terms of sensory axonal function, MAGE correlated with minimum I/V slope (r= 0.79), SDTC (r= 0.62) and latency (r=0.87) (P<0.05). Cumulatively, these findings suggest that glycaemic variability rather than

acute blood glucose is an important mediator of axonal dysfunction in type 1 diabetes.

INTRODUCTION

The finding of the previous chapter suggested that type 1 diabetic patients treated with CSII had relatively normal axonal excitability whilst patients treated with MDII had more pronounced abnormalities in axonal excitability (Kwai *et al.*, 2014). These findings were noted in groups that were matched for clinical characteristics that may affect axonal function such as disease duration, HbA1c%, BMI and insulin dosage.

Glycosylated haemoglobin (HbA1c%) is the gold standard for assessing long-term glycaemic control in a diabetic population (Expert Committee on the Diagnosis and Classification of Diabetes, 2003). However, previous studies have demonstrated that axonal dysfunction, both clinical and subclinical, may occur even in patients with good glycaemic control according to current targets for HbA1c% (Misawa *et al.*, 2004) . This is in agreement with large cohort studies such as the DCCT and EDIC studies, in which strict glycaemic control was not sufficient to curtail the development of DPN in type 1 diabetic patients (Pop-Busui *et al.*, 2010).

Glycaemic control may also be assessed as a measure of glucose variability or fluctuation although this assessment has not been implemented into routine clinical practice (Hirsch and Brownlee, 2005, Bragd *et al.*, 2008, Monnier and Colette, 2008). Glucose variability has been linked to oxidative stress and subsequent endothelial dysfunction and may contribute to the development of microvascular complications (Kilpatrick *et al.*, 2006, Bruttomesso *et al.*, 2008).

Glucose variability may be assessed as the mean amplitude of glycaemic excursions (MAGE) (Monnier *et al.*, 2008). This provides an objective measure of glucose variability over a 48-hour period, which integrates only significant positive and

negative glucose fluxes (Service *et al.*, 1970, Siegelaar *et al.*, 2010). Calculating MAGE has been made markedly easier with the recent development of continuous glucose monitoring systems (Klonoff, 2005, Monnier *et al.*, 2008). Such systems are more widely used to tailor clinical treatment of diabetes by allowing clinicians to assess diurnal and nocturnal blood glucose excursions (Monnier *et al.*, 2008). The potential relationship between glucose variability and axonal function in a human setting has not yet been explored. The aim of the present study was thus to explore the hypothesis that glucose variability, measured as MAGE, was related to axonal dysfunction in type 1 diabetes.

METHODS

Twelve patients type 1 diabetic patients (3M:9F) were recruited from Prince of Wales Hospital. All patients had been diagnosed with diabetes for at least 12 months and had been on their respective insulin regimen either CSII (n=8) or MDII (n=4) for a minimum of 12 months. All patients underwent continuous glucose monitoring for a week using an Enlite™ sensor and Minilink™ transmitter (Medtronic MiniMed, Inc., Northridge, CA). The system was paired with either the Guardian ® REAL-Time or Paradigm ® Veo ™ (Medtronic, Northridge, Northridge, CA) which allowed the fast and accurate acquisition of projected blood glucose results during the testing period. Sensors were inserted subcutaneously approximately 10cm away from the umbilicus. For patients treated with CSII, sensor implantation occurred at least 10cm away from infusion set site in addition to the umbilicus. The sensor allowed for rapid data acquisition regarding glucose levels, extrapolated from samples taken. Glucose variability was measured as mean amplitude of glycaemic excursions (MAGE) and calculated using well established methods (Service *et al.*, 1970, Monnier *et al.*, 2007, Monnier *et al.*, 2008). Essentially, the mean value of blood glucose fluctuation over the course of a 48-hr period at the time of testing was taken for consecutive peak to trough (nadir) or trough to peak (depending on which occurred first during the sample period). As per original methodology, MAGE calculations only regarded glucose fluxes of >1SD (Service *et al.*, 1970).

Testing schedule and procedure

The following schedule of neurophysiological assessment was implemented.

The first day of testing involved recruitment and consent from patients. Comprehensive neurological examinations were performed on all patients. Examination included assessment of pinprick (NeurotipTM, Owen Mumford, United Kingdom) and vibration (128Hz), reflex assessments and symptom report. Additionally, sural and tibial NCS were also undertaken to rule out patients with neuropathy. Patients were briefed on this day about the study timeline. On the second day of testing, motor and sensory axonal excitability studies were performed at three different acute glucose ranges: low (<4mmol/L), medium (4 – 10mmol/L) and high (>10mmol/L). These were measured using finger-stick measurement of blood glucose. Sensor insertion and calibration occurred after the first set of axonal excitability assessments and patients were briefed on how to perform calibrations using either the Guardian system (if they did not have an appropriate pump to transmit sensor signals to) or their insulin pump. Follow-up testing occurred a week later whereby sensors were removed and either the Guardian monitor or patient pumps were downloaded using the Medtronic Carelink program (Medtronic, Northridge, Northridge, CA). Continuous glucose monitor reports were then obtained and clinically notable reports including frequency of hypoglycaemic events, diabetic ketoacidosis and sensor failure were documented.

Statistical analysis

Shapiro Wilk assessments were used to determine the normality of data. To determine axonal excitability abnormalities, patient data taken at mid-range blood glucose was compared to age and sex matched normal control subjects using either Student's t tests or Mann Whitney U tests where appropriate (SPSS statistics software v. 22, IBM, Chicago, IL). To compare the effect of acute glucose levels on axonal excitability over the course of a day, patient group data taken at low, medium and high range blood glucose was compared using mixed model analysis as blood glucose levels in two patients failed to reach the low range (<4mmol/L) over the day of testing. The relationship between axonal excitability parameters and HbA1c%, immediate blood glucose levels and disease duration was determined using either Pearsons or Spearman Rho correlations where appropriate. To establish the relationship between axonal excitability and glucose variability, correlations were performed on patient data taken at mid blood glucose levels and MAGE.

RESULTS

Clinical characteristics

A total of 12 patients (26 ± 2.07years old) were recruited and tested. As a group, the patients had a mean HbA1c% of 8.03±0.44% and BMI of 27.78±1.75kg/m^2. Additionally, all patients had normal sural and tibial nerve amplitudes (19.50±1.52 μV and 15.79 mV respectively) and presented with no signs or symptoms of neuropathy. Continuous glucose monitoring was well tolerated by all patients. Average mean amplitude of glycaemic excursions (MAGE) for the group was 7.91 ± 0.49 mmol/L and was higher in the MDII (9.22 ± 1.02 mmol/L) group than the CSII group (7.42 ± 0.49) although this difference was not statistically significant *(P*=0.60*).*

Excitability findings at three glucose ranges

To assess the effect of acute blood glucose on axonal function, excitability studies were performed three times over the course of the day on the patients at high (>10mmol/L), medium (4-10mmol/L) and low (<3mmol/L) blood glucose levels. These readings were compared to age matched normal controls (n=21).

Type 1 diabetic patients had abnormal motor and sensory axonal excitability at all three ranges. Specifically, motor studies taken within the high range of blood glucose indicated prominent reductions in threshold change during threshold electrotonus and the recovery cycle paradigms at multiple time points (Figure 4. 1). In threshold electrotonus, reductions in percentage change in threshold occurred during S2 Accommodation (patients 19.10±0.85, controls 23.58±0.09, *P*<0.01) and undershoot (patients -15.36±0.50, controls -19.19±0.77, *P*<0.01). During the recovery cycle,

reduced threshold change was noted in superexcitability (patients -21.69±1.53, controls -26.21±1.03, $P<0.05$), superexcitability at 5ms (patients -16.96±3.91, controls 26.30±1.02) and prominently subexcitability (patients 10.14±1.03, controls 15.42±1.18, $P<0.01$). Sensory axonal function was also abnormal in the patients at the higher range (Figure 4. 1 and Table 4. 2) such that similar reductions in threshold change were present. In threshold electrotonus Accommodation half time (high 36.99±2.31, controls 30.68±1.62, $P<0.05$), undershoot (high -16.47±1.46, controls -19.70±0.80, $P<0.05$) and TEh(10-20ms) (high -77.33±2.39, controls -78.36±0.99, $P<0.05$) was reduced. Additionally, mean recovery cycle data for patient sensory axons had simultaneously a prominent reduction in subexcitability (high 7.27± 0.60, controls 10.30± 0.30, $P<0.01$).

Alterations in excitability also occurred when patients were tested at medium and low range blood glucose levels. Minimum I/V slopes (low 0.27±0.02, controls 0.26±0.01, $P<0.05$). In threshold electrotonus, reduced S2 Accommodation (medium 19.31±0.88, $P<0.01$; low 20.74±1.34, $P<0.05$), TEh(90-100ms) (medium -107.23±3.47, controls -120.50±4.07, $P<0.05$) and undershoot (medium -16.56±1.44, low -15.78±1.39, $P<0.05$) were noted. During the recovery cycle, reduced threshold change in superexcitability (medium -22.89±1.11, $P<0.05$), superexcitability at 5ms (low -20.50±4.11, $P<0.05$) and prominently subexcitability (medium 10.37±0.96, $P<0.01$; low 11.63±1.63, $P<0.05$) was noted compared to controls.

Similarly, sensory axonal findings at medium and low ranges were also abnormal compared to controls. An increase in hyperpolarizing I/V (low 0.40±0.04, $P<0.05$) was noted. This occurred in concert with greater accommodation half time (medium 37.63±2.03, low 38.35±3.17, $P<0.05$), reduced TEh(10-20ms) (medium -

73.33±2.09, low -75.83±2.45, $P<0.05$) and undershoot (medium -16.96±1.23, low -16.35±0.87, $P<0.05$) when compared to controls.

Table 4. 1. Median motor excitability comparison: type 1 diabetic patients at different blood glucose levels vs controls

MOTOR	*Patients per blood glucose level*			
Excitability parameters	*High*	*Medium*	*Low*	*Controls*
Current threshold				
Minimum I/V slope	0.23 ± 0.01	0.25 ± 0.01	0.27 ± 0.02*	0.26 ± 0.01
Recovery Cycle				
Superexcitability	-21.69 ± 1.53*	-22.89 ± 1.11*	-24.11 ± 22.01	-26.21 ± 1.03
Superexcitability (@5ms)	-16.96 ± 3.91*	-17.96 ± 4.71	-20.50 ± 4.11*	-26.30 ± 1.02
Subexcitability	10.14 ± 1.03*	10.37 ± 0.96**	11.63 ± 1.63*	15.42 ± 1.18
Threshold electrotonus				
S2 Accommodation	19.10 ± 0.85**	19.31 ± 0.88**	20.74 ±1.34*	23.58 ± 0.09
TEh(90-100ms)	-112.53 ± 3.76	-107.23 ± 3.47*	-112.53 ± 2.79	-120.50 ± 4.07
TEd(undershoot)	-15.36 ± 0.50**	-16.56 ± 1.44*	-15.78 ± 1.39*	-19.19 ± 0.77

Mean group motor data for each blood glucose level ± SEM. Excitability parameters are expressed as % change in threshold. Blood glucose levels are in terms of mmol/L values whereby low = <4mmoL/L, medium = 4-10mmol/L and high = 10+mmol/L. Analysis revealed that patient excitability was abnormal at low, mid and high blood glucose levels when compared to normal controls. *P<0.05 and **P<0.01.

Table 4. 2. Median sensory excitability comparison: type 1 diabetic patients at different blood glucose levels vs controls

SENSORY	*Patients per blood glucose level*			
Excitability parameters	*High*	*Medium*	*Low*	*Controls*
Current threshold				
Hyperpolarizing I/V	0.38 ± 0.03	0.34 ± 0.02	0.40 ± 0.04*	0.32 ± 0.01
Recovery Cycle				
Subexcitability	7.27 ± 0.60**	7.24 ± 0.66*	8.56 ± 0.3*	10.30 ± 0.30
Threshold electrotonus				
Accommodation (half time)	36.99 ± 2.31*	37.63 ± 2.03*	38.35 ± 3.17*	30.68 ± 1.62
TEh(10-20ms)	-77.33 ± 2.39*	-73.33 ± 2.09*	-75.83 ± 2.45	-78.36 ± 0.99
TEd(undershoot)	-16.47 ± 1.46*	-16.96 ± 1.23*	-16.35 ± 0.87*	-19.70 ± 0.80

Mean group sensory data for each blood glucose level ± SEM. Excitability parameters are expressed as % change in threshold. Blood glucose levels are in terms of mmol/L values whereby low = <4mmoL/L, medium = 4-10mmol/L and high = 10+mmol/L. Analysis revealed that patient excitability was abnormal at low, mid and high blood glucose levels when compared to normal controls . *P<0.05 and **P<0.01.

Acute glucose levels did not have an effect on axonal function

Given that patient excitability was abnormal at the different glucose ranges, mean data for the three levels were compared using a mixed model analysis (two patients did not reach low range on the day of testing). As a group, mixed model analysis revealed that there were no significant differences in axonal excitability parameters between the three acute glucose ranges (Table 4. 3, Table 4. 4 and Figure 4. 1). Specifically, for motor studies (Table 4. 3) no significant differences were noted in threshold electrotonus parameters; these included S2 Accommodation, undershoot, TEd(10-20ms) and peak response in addition to TEh(10-20ms) and TEh(20-40ms). Likewise, no significant differences were noted for recovery cycle parameters super and subexcitability nor the current threshold parameter hyperpolarizing I/V slope was also not significantly different between the groups.

Similarly, mean data for sensory axonal function also exhibited no differences between the glucose levels (Table 4. 4). Again, threshold electrotonus parameters S2 Accommodation, and responses to early hyperpolarizing (at 10-20ms) pulses and depolarizing pulses (at 90-100ms and undershoot phase) were not different between the levels ($P>0.05$). Additionally, recovery cycle parameters, superexcitability and subexcitability which prominently appeared abnormal in type 1 diabetic patients early in this thesis, were not different at the different blood glucose levels. Likewise, current threshold relationship parameters minimum I/V slope and hyperpolarizing I/V slope were similar between the blood glucose groups.

Additionally, correlative analysis between these parameters and blood glucose via finger-stick at the time did not find any associations with the parameters prominently shown to be abnormal in these patients (Figure 4. 2 and Figure 4. 3). Specifically in

motor studies, blood glucose levels via finger-stick did not correlate with minimum I/V (r=-0.02 and *P*=0.94), undershoot (r=0.35 and *P*=0.29), superexcitability (r=0.10 and *P*=0.75) and subexcitability (r=-0.41 and *P*=0.18). Sensory studies likewise found no correlations with these parameters: Minimum I/V (r=0.12 and *P*=0.72), undershoot (r=-0.08 and *P*=0.80), superexcitability (r=-0.20 and *P*=0.54) and subexcitability (r=-0.37 and *P*=0.29). These findings suggest that acutely, blood glucose levels do not have an effect on peripheral axonal function.

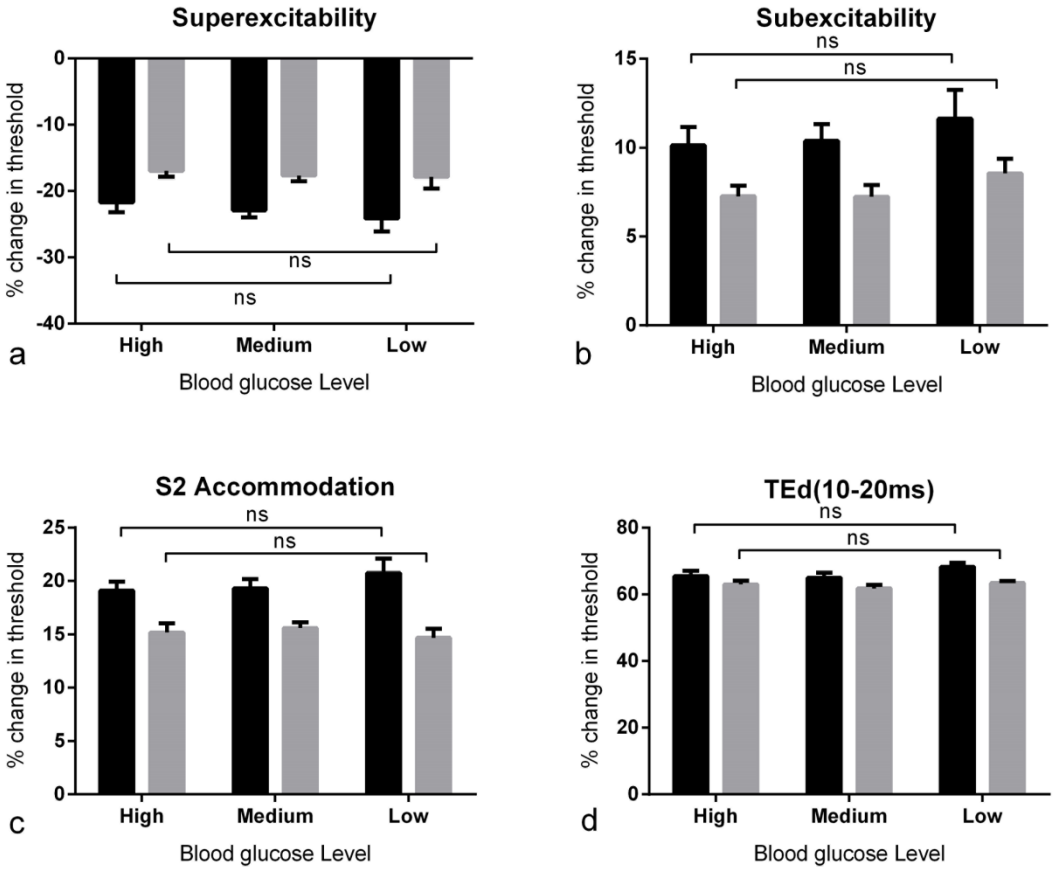

Figure 4. 1. Mean superexcitability, subexcitability, S2 Accommodation and TEd(10-20ms) at different glucose ranges

Superecitabilty, subexcitability, S2 Accommodation and TEd(10-20ms) (a,b,c and d resectively) were non significantly different between high, medium and low blood glucose levels in the group for either motor (black) or sensory (grey) recordings. Values are expressed as % change in threshold with SEM

Table 4. 3. Median motor excitability comparison between blood glucose levels

MOTOR	*Patients per blood glucose level*			
Excitability parameters	*High*	*Medium*	*Low*	*P value*
Stimulus response				
Stimulus response slope	5.47 ± 0.38	5.89 ± 1.07	5.82 ± 105	*0.13*
Current threshold				
Minimum I/V slope	0.23 ± 0.01	0.25 ± 0.01	0.27 ± 0.02	*0.15*
Recovery Cycle				
Superexcitability	-21.69 ± 1.53	-22.89 ± 1.11	-24.11 ± 22.01	*0.56*
Superexcitability (@5ms)	-16.96 ± 3.91	-17.96 ± 4.71	-20.50 ± 4.11	*0.96*
Subexcitability	10.14 ± 1.03	10.37 ± 0.96	11.63 ± 1.63	*0.66*
Threshold electrotonus				
S2 Accommodation	19.10 ± 0.85	19.31 ± 0.88	20.74 ±1.34	*0.47*
TEd(10-20ms)	65.42 ± 1.61	65.03 ± 1.53	68.31 ± 1.18	*0.70*
TEh(90-100ms)	-112.53 ± 3.76	-107.23 ± 3.47	-112.53 ± 2.79	*0.76*
TEd(undershoot)	-15.36 ± 0.50	-16.56 ± 1.44	-15.78 ± 1.39	*0.44*

Mean group motor data for each blood glucose level ± SEM. Excitability parameters are expressed as % change in threshold. Blood glucose levels are in terms of mmol/L values whereby low = <4mmoL/L, medium = 4-10mmol/L and high = 10+mmol/L. Analysis revealed that mean excitability of the patients was not significantly different between high, medium and low blood glucose levels over a day of testing. Significance was considered when $P<0.05$

Table 4. 4. Median sensory excitability comparison between blood glucose levels

SENSORY	*Patients per blood glucose level*			
Excitability parameters	*High*	*Medium*	*Low*	*P value*
Current threshold				
Hyperpolarizing I/V	0.38 ± 0.03	0.34 ± 0.02	0.40 ± 0.04	*0.53*
Recovery Cycle				
Subexcitability	7.27 ± 0.60	7.24 ± 0.66	8.56 ± 0.30	*0.38*
Threshold electrotonus				
Accommodation (half time)	36.99 ± 2.31	37.63 ± 2.03	38.35 ± 3.17	*0.98*
TEd(10-20ms)	65.47 ± 1.61	65.03 ± 1.53	68.31 ± 1.18	*0.70*
TEh(10-20ms)	-77.33 ± 2.39	-73.33 ± 2.09	-75.83 ± 2.45	*0.60*
TEd(undershoot)	-16.47 ± 1.46	-16.96 ± 1.23	-16.35 ± 0.87	*0.94*

Mean group sensory data for each blood glucose level ± SEM. Excitability parameters are expressed as % change in threshold. Blood glucose levels are in terms of mmol/L values whereby low = <4mmoL/L, medium = 4-10mmol/L and high = 10+mmol/L. Analysis revealed that mean excitability of the patients was not significantly different between high, medium and low blood glucose levels over a day of testing. Significance was considered when a $P<0.05$.

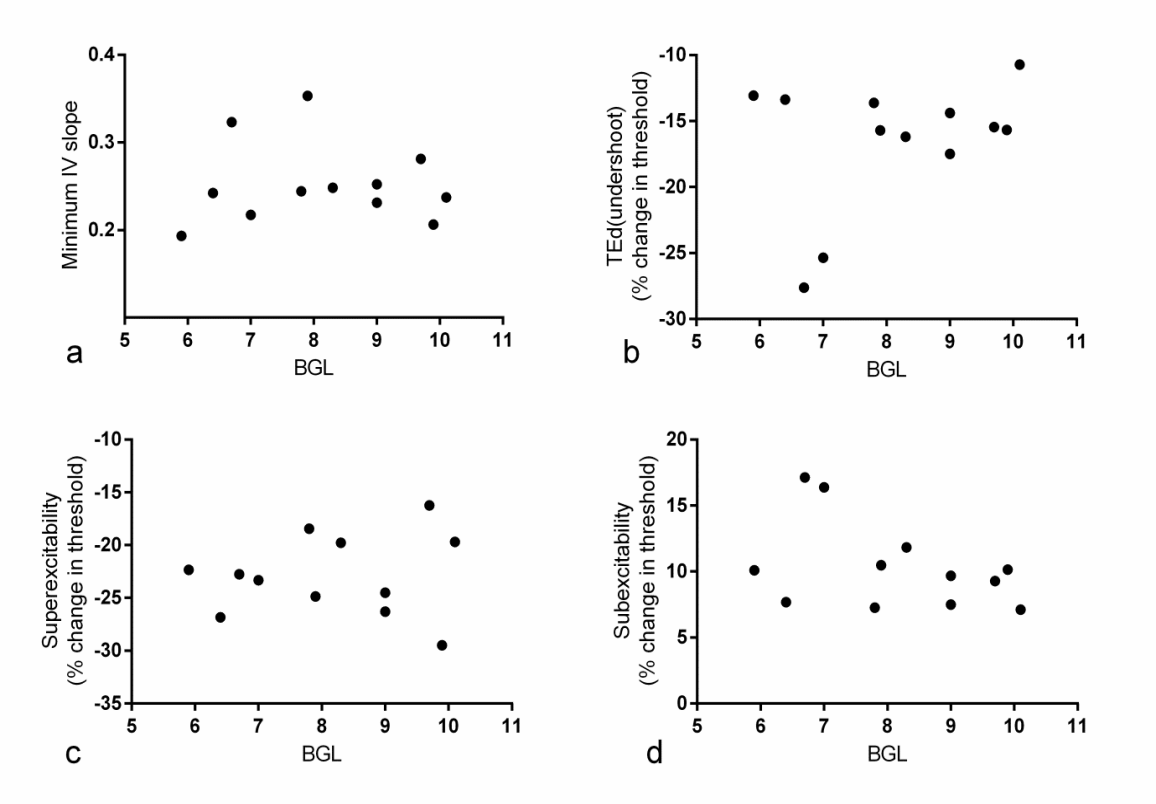

Figure 4. 2. Correlation between acute blood glucose and motor excitability parameters

Correlations between motor excitability parameters minimum I/V (a), TEd(undershoot) (b), superexcitability (c) and subexcitability (d) and blood glucose level as measured by finger-stick (BGL). No significant correlation was noted between these parameters and blood glucose level. Significance was considered when $P<0.05$.

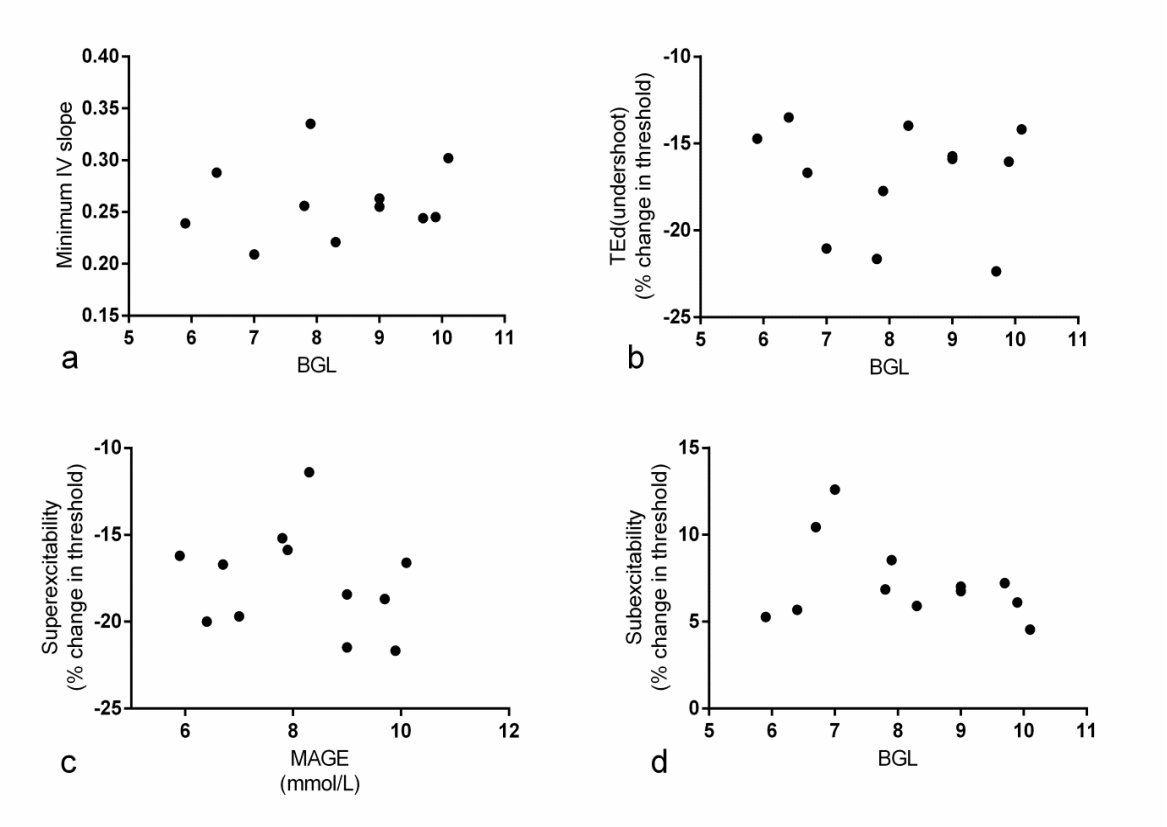

Figure 4. 3. Correlation between acute blood glucose and sensory excitability parameters

Correlations between sensory excitability parameters minimum I/V (a), TEd(undershoot), (b) superexcitability, (c) and subexcitability, (d) and blood glucose level as measured by finger-stick (BGL). No significant correlation was noted between these parameters and blood glucose level. Significance was considered when $P<0.05$.

Glucose variability correlated with impaired axonal function

Given that acute blood glucose concentrations did not correlate with neurophysiological parameters, glucose variability analysis was undertaken to assess the effect of fluctuating glucose on axonal function, using continuous glucose monitoring and mean amplitude of glycaemic excursion (MAGE) measurements. A single MAGE value was calculated for each patient as a measure of glucose variability.

For motor recordings taken at mid range blood glucose (Figure 4. 4), higher MAGE readings correlated with changes in excitability parameters that have been previously shown to be abnormal in diabetic patients (Misawa *et al.*, 2005, Sung *et al.*, 2012, Arnold *et al.*, 2013). Specifically, there was correlation between MAGE and superexcitability at 7ms (r=0.62 and *P*=0.04), minimum I/V slope (r=0.89 and *P*=0.001) and with refractoriness at 2ms (r=0.78 and *P*=0.06). MAGE also correlated with motor excitability parameters at the higher range of blood glucose such that greater MAGE was associated with less S2 Accommodation (r=-0.71 and *P*=0.02) and TEd(40 Accommodation) (r=-0.70 and *P*=0.02). There was also a reduction in sueperxcitability with higher MAGE readings however this did not reach statistical significance (r=0.63 and *P*=0.05).

For sensory axonal excitability studies (Figure 4. 5), higher MAGE readings correlated with strength duration time constant (r=0.79 and *P*=0.01) a marker of Na_p. Additionally, similar to motor responses, greater MAGE correlated with minimum I/V slope (r=0.62 and *P*=0.05) and a reduction in threshold change was also found in superexcitability (r=0.53 and *P*=0.09) however this did not reach statistical

significance. Notably, MAGE also correlated with latency when taken at the higher blood glucose levels (r=0.90 and $P<0.001$).

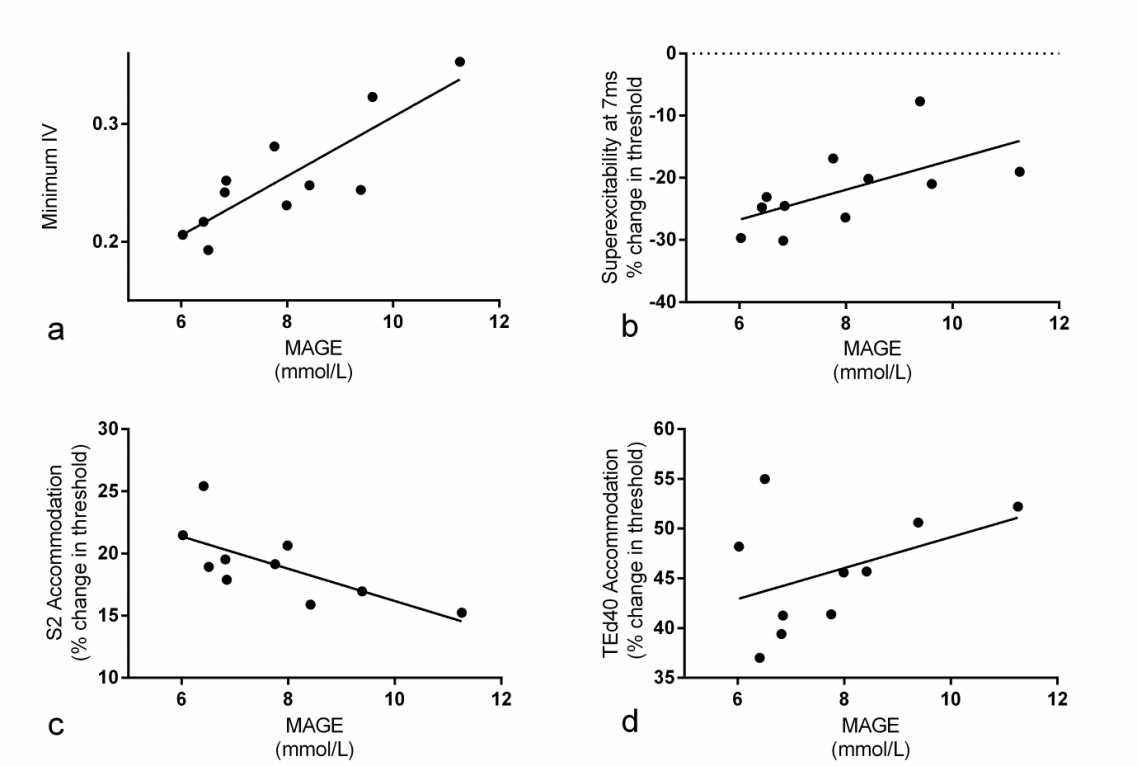

Figure 4. 4. Mean amplitude of glycaemic excursions correlated with motor excitability

Mean amplitude of glycaemic excursions correlated with motor axonal excitability parameters. MAGE is expressed in mmol/L. MAGE was strongly and positively correlated with (a) minimum I/V (r=0.89 and *P*=0.001) and a reduction in absolute superexcitability at 7ms (b) after supramaximal stimuli (r=0.62 and *P*=0.04). Increased MAGE also correlated with (c) less S2 Accommodation within the higher range (r=-0.71 and *P*=0.02) and (d) increased TEd 40Accommodation (r=-0.70 and *P*=0.02).

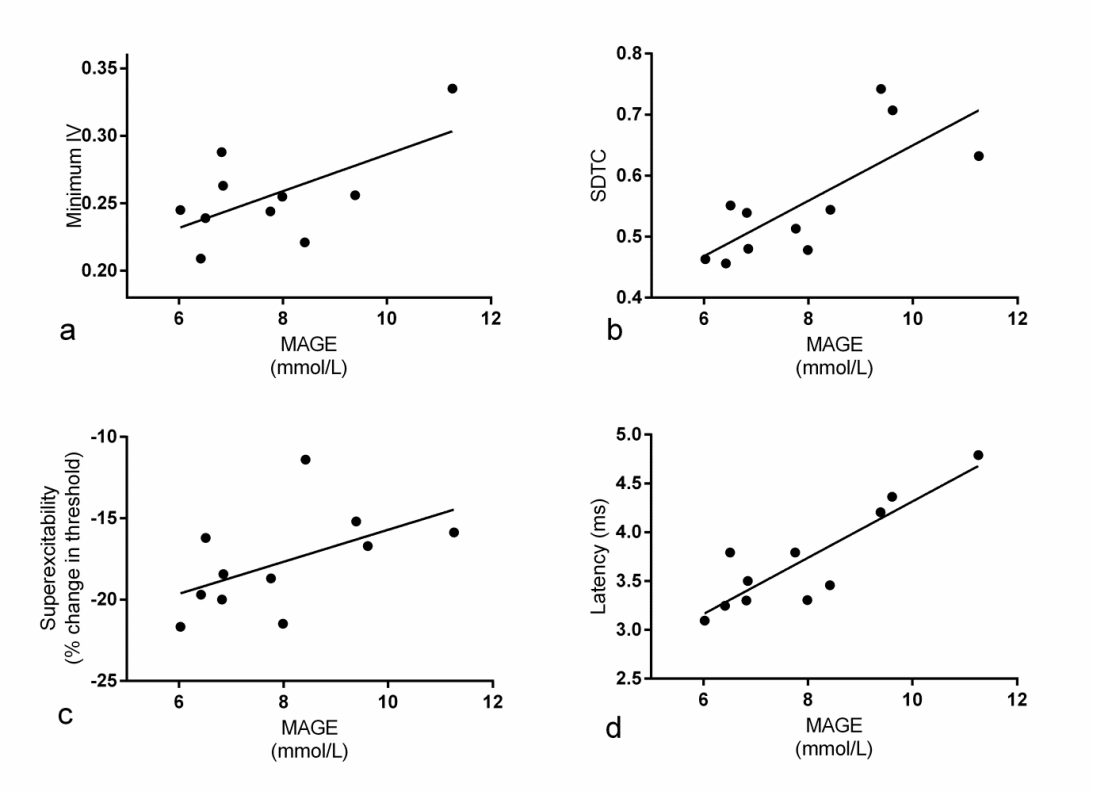

Figure 4. 5. Mean amplitude of glycaemic excursions correlated with sensory excitability

Mean amplitude of glycaemic excursions (MAGE) correlated with sensory axonal excitability. MAGE is expressed as mmol/L. Minimum current threshold slope (Minimum I/V) (a) and strength-duration time constant (SDTC) (b) correlated positively with greater glycaemic variability expressed as MAGE (r= 0.79, P=0.01; r= 0.62, P=0.05 respectively). An increase in superexcitability was also noted although this did not reach statistical significance (c) (r=0.53, P=0.09). At higher range, MAGE also correlated with latency (d) (r=0.87 and P<0.01).

DISCUSSION

The findings of the present study indicate that glucose fluctuation, measured using mean amplitude of glycaemic excursions (MAGE) rather than acute glucose levels are associated with altered axonal function in type 1 diabetic patients. The finding has important clinical implications as it suggests that monitoring of glycaemic control through either acute blood glucose concentrations or by means of HbA1c% may be insufficient in preventing the development of axonal dysfunction.

The impact of hyperglycaemia has been previously explored in a cross sectional study by Misawa and colleagues (2005) who found that higher HbA1c% was associated with less superexcitability and subexcitability (Misawa *et al.*, 2005). Additionally, the same authors found that hyperglycaemia as measured by HbA1c% was associated with shorter refractory periods (Misawa *et al.*, 2004, Misawa *et al.*, 2006). Those studies differ from the present study as patients with both type 1 and type 2 diabetes were included and some patients in those studies had evidence of overt neuropathy. Recruitment in the present study was restricted to type 1 diabetic patients and given the potential differences in the underlying mechanisms of neuropathy between type 1 and type 2 diabetes (Sima *et al.*, 1986, Sima *et al.*, 1988), it is possible that the relationship of acute glucose levels to axonal dysfunction may be different in a type 2 diabetic cohort.

Specific to type 1 diabetes, findings from the DCCT and subsequent EDIC studies demonstrated that glycaemic control via tight regulation of HbA1c% is insufficient to curtail the development of neuropathy at long term follow-up (Pop-Busui *et al.*, 2010). Although it has been proposed that underlying metabolic aberrations such as C-peptide deficiency contribute to axonal dysfunction in type 1 diabetes (Grunberger

et al., 2001, Sima *et al.*, 2001, Sima, 2003), recent studies have suggested that glucose variability may also play a role due to its effects on cellular metabolism (Brownlee, 2001, Sun *et al.*, 2012). Schwann cells undergo activation of apoptotic pathways in the presence of intermittent high glucose (Sun *et al.*, 2012). These were hypothesized to be linked to oxidative stress, which plays a crucial role in unifying the multiple proposed pathways involved in development of diabetic complications including neuropathy, retinopathy and nephropathy (Brownlee, 2001, Brownlee, 2005). A study by Monnier and colleagues (2006) described a correlation between MAGE and urinary excretion of 8-iso prostaglandin $F2_{\alpha}$ (Monnier *et al.*, 2006), an indicator of oxidative stress that is derived from free radical mediated oxidation of arachidonic acid (Morrow *et al.*, 1990, Ceriello, 2003, Ceriello, 2005). This metabolite has been shown to increase in the presence of diabetes and decrease with control of postprandial glucose spikes (Trovati *et al.*, 1991, Monnier *et al.*, 2006). Interestingly, on a more acute level, the relationship between this marker and variability in blood glucose is strengthened when hypoglycaemic type 1 diabetic patients are quickly treated to a hyperglycaemic state (Ceriello *et al.*, 2014).

From a neurological perspective, clinical studies have found that greater glucose spikes occurred in diabetic patients with painful neuropathy (Oyibo *et al.*, 2002, Cao *et al.*, 2009). In the study by Cao and colleagues (2009), MAGE recordings were higher in diabetic patients with severe painful neuropathy compared to those with mildly painful neuropathy (Cao *et al.*, 2009). This study also found that HbA1c% was non-significantly different between these groups. Early studies in healthy controls utilizing hyperglycaemic clamps have suggested that acutely, glucose fluctuation may be associated with autonomic axonal dysfunction (Yeap *et al.*,

1996). These manifested as increased supine heart rates and were hypothesized to be due to the biochemical effects of glucose on parasympathetic axonal function.

The present study suggested that higher MAGE is associated with reduced threshold change in superexcitability, increased latency, a reduction in threshold change in threshold electrotonus, including S2 Accommodation and a rightward shift or increase in minimum I/V slope during the current threshold relationship (Figure 4. 4 and Figure 4. 5). These changes are suggestive of an ischaemic depolarization via Na^+/K^+ pump dysfunction (Kiernan and Bostock, 2000) which is well established in the pathogenesis of DPN (Greene, 1986, Edwards *et al.*, 2008). A possible mechanism for this outcome in the presence of a higher MAGE may be impairment of neurovascular function. Glucose variability is associated with oxidative stress and endothelial dysfunction (Buscemi *et al.*, 2010, Schisano *et al.*, 2011). As the changes in excitability are consistent with alteration to energy dependent processes (see Chapter 2), it is possible that greater glucose variability in type 1 diabetes impairs neurovascular function. Ensuing energy depravation of the Na^+/K^+ pump may contribute to axonal depolarization and could explain the phenotype described in the present study. It is therefore possible that patients with higher MAGE undergo greater oxidative stress that potentiates a cascade of metabolic events leading to Na^+/K^+ pump dysfunction and altered axonal excitability.

In terms of acute blood glucose effects on axonal function, our findings suggested that there were no differences in axonal excitability between high, medium and low blood glucose ranges. A potential explanation may be that the blood glucose ranges were not wide enough to capture the adverse effect of extremely high or low glucose levels on axonal function. Animal studies have indicated alterations in axonal

metabolism only at glucose levels well outside normal physiological ranges (<2 mmol/L and >29mmol/L)(Stecker and Stevenson, 2014). The present study did not assess the effects of these extremes of glucose concentration, but rather sought to address the effects over a range of glucose levels that are more commonly encountered in a clinical setting.

From a clinical perspective, the findings of the present study suggest that blood glucose variability may play a crucial role in axonal dysfunction and thus neuropathy development in a type 1 diabetic cohort. Cumulatively, these data suggest that controlling glucose variability may be an additional step to preventing neuropathy development in a type 1 diabetic patient cohort.

Summary and Conclusions

SUMMARY OF THESIS

The studies comprising this thesis have explored the pathophysiology of axonal dysfunction in diabetes using axonal excitability techniques. Abnormalities of axonal excitability have been demonstrated in both type 1 and type 2 diabetic patients with and without signs of neuropathy. Furthermore, excitability markers have been linked to measures of neuropathy-specific QoL and alterations in axonal function were apparent in non-neuropathic type 2 diabetic patients, indicating that alterations in axonal excitability findings may precede clinical signs of neuropathy in this population. Axonal dysfunction was further extended to non-neuropathic type 1 diabetic patients who are vulnerable to a more severe phenotype of DPN. These studies indicated a window of opportunity for neuroprotective intervention and thus the following series of studies characterized the potential neuroprotective role of current insulin regimens, and importantly provided evidence for glycaemic variability as a contributor to axonal damage in diabetic states.

In Chapter 1, the effects of axonal ion channel dysfunction on neuropathy-specific quality of life (QoL) measures was explored. Fifty-four patients with type 2 diabetes underwent comprehensive neurological assessment, NCS and axonal excitability assessment. Neuropathy severity was assessed using the Total Neuropathy Score. Neuropathy-specific QoL was assessed using the neuropathy-specific-QoL questionnaire (NeuroQoL). NeuroQoL scores indicated significant QoL impairment (mean=9.08$\pm$5.93). Strength-duration time constant (SDTC), an excitability parameter reflecting Na^+ channel function, was strongly correlated with QoL scores (r=0.545, P<0.005). SDTC was prolonged in 48.6% of patients who experienced neuropathic symptoms. A significant correlation was also noted between SDTC and neuropathy severity (r = 0.29, P <0.05). This relationship was strengthened when

looking specifically at patients with clinically graded neuropathy (r = 0.366, *P* <0.05). The results of the study demonstrated an association between markers of Na^+ channel function and QoL in DPN. The study demonstrates that excitability techniques may identify patients in whom altered Na^+ channel function may be the dominant abnormality. The findings suggest that excitability techniques may have a role in clinical decision making regarding neuropathic pain management.

Once axonal dysfunction, specifically ion channel dysfunction, had been characterized in type 2 diabetic patients, Chapter 2 assessed patients with type 1 diabetes in order to provide insights into the mechanisms of axonal dysfunction in this patient cohort.

In Chapter 2, sensory and motor axonal excitability studies were undertaken in thirty type 1 diabetic patients. Compared to controls, type 1 diabetic patients demonstrated significant axonal excitability abnormalities in both sensory and motor nerves, indicating axonal membrane depolarization. Mathematical modelling results demonstrated that these changes were due to reduced nodal Na^+ conductance, nodal/paranodal K^+ conductances and Na^+/K^+ pump dysfunction. Changes in these conductances are consistent with axo-glial dysjunction as outlined in animal models of type 1 diabetes. These were found in the absence of DPN. Accordingly, the study provided support for functional alterations in axons of type 1 diabetic patients early in the disease. These data indicate a window of opportunity for prophylactic intervention and provide a platform for investigating concomitant C-peptide infusion as a neuroprotective measure in type 1 diabetes.

The findings of Chapter 2 suggested that axonal dysfunction occurred in type 1 diabetic patients in the absence of clinical signs and symptoms of neuropathy and are

suggestive of a window of opportunity for neuroprotective treatment in type 1 diabetic patients. Although strict glycaemic control may reduce the risk of developing DPN, the neurological benefits of different insulin regimens remained relatively unknown.

Chapter 3 explored the neuroprotective potential of current insulin regimens for type 1 diabetic patients. Fifty-one consecutive patients with type 1 diabetes underwent clinical neurological assessment. Subsequently, 41 non-neuropathic patients, 24 of whom were receiving MDII, and 17 receiving CSII, underwent motor axonal excitability testing. Treatment groups were matched for glycaemic control, BMI, disease duration and gender and neurophysiological parameters were compared between treatment groups and those taken from age and sex matched normal controls. MDII-treated patients manifested prominent abnormalities when compared to normal controls in threshold electrotonus properties including TEd(10-20ms), undershoot and TEh(90-100ms) ($P<0.05$). Additionally, recovery cycle parameters superexcitability and subexcitability were also abnormal ($P<0.05$). In contrast, axonal function in CSII-treated patients was within normal limits when compared to age matched controls. The differences between the groups was noted in cross-sectional analysis and remained at longitudinal follow-up. These findings indicated that axonal function in type 1 diabetes is maintained within normal limits in patients treated with CSII and not MDII. The data also raised the possibility that CSII therapy may have neuroprotective potential in patients with type 1 diabetes. The mechanisms for this finding remained unclear. It is possible that differences are due to glycaemic variability which has been put forward as a benefit of CSII therapy.

In Chapter 4, the role of glycaemic variability and acute glucose levels on motor and sensory axonal dysfunction in diabetic patients was explored, utilizing continuous glucose monitoring and axonal excitability techniques at different blood glucose ranges. Axonal excitability studies were undertaken in 12 type 1 diabetic patients. Median motor and sensory axonal excitability studies were obtained at high (>10mmol/L), medium (4-10mmol/L) and low (<4mmol/L) blood glucose ranges in all patients on a single day. Mean amplitude of glycaemic excursions (MAGE), a measure of glycaemic variability, was assessed in all patients over a 48-hour period using a subcutaneous glucose sensor. Patient data showed reduced threshold change in threshold electrotonus, recovery cycle and current threshold relationships as noted in previous chapters at all glucose ranges. Mixed model analysis revealed no significant differences between high, medium and low range mean excitability data for either motor or sensory studies. Importantly, mean amplitude of glycaemic excursions (MAGE), a measure of glycaemic variability, was strongly correlated with motor excitability parameters including minimum I/V slope (r= 0.89), superexcitability (r=0.62), S2 Accommodation (r=-0.71) and TEd(40Accommodation) (r=-0.70) (P<0.05). In terms of sensory axonal function, MAGE correlated with minimum I/V slope (r= 0.79), SDTC (r= 0.62) and latency (r=0.87) (P<0.05). Cumulatively, these findings suggested that glycaemic variability rather than acute blood glucose is an important mediator of axonal dysfunction in type 1 diabetes.

PERSPECTIVES FOR FUTURE WORK

The studies in this thesis have shown that axonal excitability abnormalities occurred in diabetic patients who had no clinical signs of neuropathy. This suggests that axonal excitability techniques may have a predictive role in stratifying patients most at risk for developing DPN. Longitudinal follow-up studies of patients in terms of neurological clinical outcomes and axonal excitability parameters are required to formally test this hypothesis. These studies would involve motor and sensory axonal excitability studies, NCS and staging of neuropathy severity to determine if clinical neuropathy development was related to greater axonal excitability abnormalities early in the disease course. These studies would direct the development of neuroprotective strategies and stratify patients according to their risk of developing DPN.

Another role for excitability techniques in the clinical realm could be a therapeutic biomarker of treatment efficacy in the management of painful DPN. Findings of Chapter 1 of this thesis found a correlation between neuropathy specific QoL as a result of ectopic symptoms and SDTC, a marker of persistent Na^+ conductances. Considering that a class of pharmacological agents for neuropathic pain management targets ion channel activity, excitabilty parameters, inculding SDTC, may potentially be used to identify patients more likely to respond to these modulators. Future studies to explore this would require a blinded study comparing changes in pain score and excitability parameters in diabetic patients before and after neuropathic pain treatment.

Chapter 3 demonstrated that there was better axonal function in CSII-treated patients over MDII-treated patients however the mechanisms underlying these differences remain unclear. It is possible that glycaemic variability may underlie the differences noted between the two treatment groups and has been put forward as a benefit of CSII therapy. Chapter 4 revealed that indeed glycaemic variability is an important determinant of axonal function in type 1 diabetes however, the relative differences between CSII and MDII in terms of glycaemic variability, and their effect on axonal function have yet to be established. A randomized clinical trial would be required to definitively establish the benefits of CSII therapy from a neurological perspective. This would be helpful in establishing whether CSII therapy is neuroprotective. After establishing this, further studies would be required to determine the cost-effectiveness of CSII therapy in long term diabetic care. Cumulatively, these studies would help to determine if CSII therapy is superior to MDII in the treatment of diabetes and importantly, in curtailing the development of DPN.

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