“An investigation of nerve excitability measures to detect the effect of ion channel active medications on peripheral nerve”

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Degree: PhD
Declaration

I, Adam James Molyneux confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed ............................................................................... 

Date ..................................................................................
Abstract

This thesis details the application of nerve excitability testing to myelinated peripheral motor and sensory nerve in order to detect and describe the effects of drugs which prior knowledge tells us act as sodium channel inhibitors. The techniques used are applicable to human subjects as well as both in-vivo and ex-vivo animal preparations. The main drug of investigation is carbamazepine. Carbamazepine has been well studied and it is used here primarily to investigate the electrophysiological techniques applied and their sensitivity to its described action.

Chapter 1 provides the historical background to the modern electrophysiological technique of threshold tracking and nerve excitability studies. The fundamentals of nervous transmission and sodium channels are described along with the history and known mechanisms of action for relevant sodium channel inhibiting compounds.

Chapter 2 provides the rationale for employing these particular techniques to investigate the effect of sodium channel blocking drugs and states two hypotheses. In this section two further hypotheses are stated which required an evolution of the existing nerve excitability techniques in order investigate. The reasoning behind this is discussed.

Chapter 3 describes the method to apply nerve excitability studies to healthy human subjects, before and after carbamazepine and rat ex-vivo saphenous nerve exposed to carbamazepine and its metabolite carbamazepine epoxide. An adaptation to the testing protocol, threshold tracking of repeated stimuli at different frequencies, is described with the intention of more fully elucidating the drug property of use or frequency-dependence.

Chapter 4 and Chapter 5 present results demonstrating that the effect of carbamazepine is detectable in both ex-vivo rat nerve and healthy human subjects with the results differing between human motor and sensory nerve.

Chapter 6 extends current electrophysiological techniques with a novel protocol designed to draw out features of sodium channel inhibition not well demonstrated by the current technique, that of frequency-dependence. Lidocaine, tetrodotoxin and carbamazepine are compared in the ex-vivo preparation. Chapter 7 provides a discussion and implications of the findings.

Chapter 8 presents an adjustment to the current mathematical model of human motor nerve in order to improve the ability to describe drugs which inhibit sodium channels. The results of implementing this change are then presented and discussed.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>CA</td>
<td>Resting channel preference</td>
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<tr>
<td>CBZ</td>
<td>Carbamazepine</td>
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<tr>
<td>CBZe</td>
<td>Carbamazepine-epoxide</td>
</tr>
<tr>
<td>CF</td>
<td>Fast inactivated preference</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CTCAE</td>
<td>Common Terminology Criteria for Adverse Events</td>
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<tr>
<td>DAP</td>
<td>Depolarising after-potential</td>
</tr>
<tr>
<td>DKa</td>
<td>Association rate constant</td>
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<tr>
<td>DKd</td>
<td>Dissociation rate constant</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
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<tr>
<td>Ih</td>
<td>Inward rectifying conductance</td>
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<td>IV</td>
<td>Current-threshold</td>
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<tr>
<td>NEP</td>
<td>Nerve excitability profile</td>
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<td>PNaN</td>
<td>Nodal sodium conductance</td>
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<td>PNS</td>
<td>Peripheral nervous system</td>
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<td>QT</td>
<td>Charge-duration</td>
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<tr>
<td>RRP</td>
<td>Relative refractory period</td>
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<tr>
<td>SAE</td>
<td>Serious adverse event</td>
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<tr>
<td>SDTC</td>
<td>Strength duration time constant</td>
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<tr>
<td>SIF</td>
<td>Synthetic interstitial fluid</td>
</tr>
<tr>
<td>SR</td>
<td>Stimulus response</td>
</tr>
<tr>
<td>SUSAR</td>
<td>Suspected unexpected serious adverse event</td>
</tr>
<tr>
<td>TEd</td>
<td>Depolarising threshold electrotonus</td>
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Chapter 1

Introduction

1.1 A brief history of peripheral nerve electrophysiology

1.1.1 From Galen to Galvani

Cobb (2002) describes how from the time of Galen in the 2nd century A.D. through to Descartes in the 17th century the idea of nerve and muscle function resulting from “spirits of motion” travelling through hollow nerves (Galen, 1549) and “animal spirits” were the proposed prime movers of the nervous system (Descartes, 1662). Despite Descartes’ breakthrough of describing human motion in mechanistic terms and providing the first description of the reflex arc, the understanding of the basis of nerve function had changed little (Figure 1.1a).

1.1.2 Swammerdam applies mechanistic thinking

It was Jan Swammerdam, a Dutch natural scientist, in the 1660s who first developed a frog nerve preparation and demonstrated that by irritation of the nerve, the muscle would contract. Irritation was performed with scissors or any other instrument. Irritation in one experiment was performed with a silver wire and the nerve fixed by a brass ring, likely resulting in a current flow (unknown at the time) being the means of stimulation. Swammerdam also tested Descartes’ hypothesis that muscles would increase in volume with contraction (Figure 1.1b). Through these experiments it was concluded that “motion or irritation of the nerve alone
is necessary to produce muscular motion”; the evidence pointed against
the theory of animal spirits, and against the theory that muscles con-
tract by inflation with air or fluid as espoused by Descartes. Swammer-
dam writings were published posthumously in 1737 and translated into
English in 1758 (Swammerdam, 1758). Jan Swammerdam applied the
mechanistic theory of Descartes in a pragmatic, experimentally testable
way, and hugely progressed thinking at the time. In his experiments,
Swammerdam noted the almost instantaneous result of irritation of the
nerve on contraction of the muscle, analogising this to the striking of a
board at one end with the almost immediate conduction of the impulse
being felt at the other coming, close to the modern understanding of ner-
vous transmission.

(a)       (b)

Figure 1.1: a) Descartes’ illustration of his hypothesis of movement of
the “animal spirits” and the reflex arc in response to burning (Descartes,
1662). b) Swammerdam’s illustration that irritation of frog nerve causes
muscle contraction (Swammerdam, 1758).

1.1.3 Galvani, Volta, Matteucci and animal electricity

Luigi Galvani followed in Swammerdam’s footsteps using the frog nerve-
muscle preparation experimentally with his wife Lucia Galeazzi and his
nephew Giovanni Aldini as co-experimenters. Galvani published De Viribus
Electricitatis in Motu Musculari Commentarius in 1791 as a summary of
his investigations. Galvani demonstrated firstly that electrical stimula-
tion of the frog nerve could cause muscular contraction, secondly that
there was a stimulus response relationship, and thirdly that there was
refractoriness on repeated stimulation (Galvani, 1791). The next major contribution came in 1794 when Galvani discovered that, when two nerve-muscle preparations were nearby and the cut nerve end of one touched either the nerve or the muscle of the other, a contraction would be elicited in the first and frequently also the second (Galvani, 1842). A review and detailed discussion of Galvani’s thinking during the period can be found in Kipnis (1987).

An opponent of Galvani’s idea that animal electricity was internal to the preparation was Alessandro Volta who successfully repeated many of Galvani’s experiments and argued that the electrical source was from outside. These investigations lead Volta to experiment with different metals to produce electricity and ultimately the Voltaic pile or forerunner of the modern day battery. After the death of Galvani, Volta’s view, that there was no intrinsic electricity within the animal, prevailed until being convincingly disproved by Carlo Matteucci, a physicist born in Forlì, Italy in 1811. Matteucci repeated experiments done by Leopoldo Nobili (1784-1835) but incorrectly interpreted, whereby a series of frog half-thighs were stacked upon one another and a current measured between the cut and uncut surfaces. Matteucci showed that the size of the current was proportional to the number of stacked half-thighs not the metal electrodes and that this current disappears during tetanus, thus providing proof of a “muscle current” or “animal electricity” (Mateucci, 1844).

1.2 Discovering the action potential

1.2.1 du Bois Reymond and Helmholtz

The first recording of the “action potential” was done by Emil du Bois-Reymond, in 1848, using a galvanometer and the experimental setups of Matteucci. This was referred to as the “negative Schwankung” or “negative variation” by du Bois-Reymond. Hermann von Helmholtz (a student of Johannes Müller) in 1850 succeeded in measuring the speed of the action potential by utilising a simple preparation and military techniques used to measure the velocity of bullets (Helmholtz, 1850). The experiment was done by stimulating the nerve and recording the latency of the muscular contraction before moving the stimulus point along the nerve and repeating the experiment, the difference in timing of the muscular
contraction in the two circumstances was deduced to be the speed at which the nerve impulse travelled between the two points of stimulation. Helmholtz then progressed to using a smoke drum to graphically record his results for publication (von Helmholtz, 1852).

1.2.2 Bernstein, Overton and ionic theory

Julius Bernstein (a student of both von Helmholtz and du Bois-Reymond) was next able to accurately measure the time course of the “negative variation” by the development of his differential rheotome. This apparatus allowed sampling of the current at different time points after a stimulus; through the use of repeated stimuli a picture could be built of the electrical process under a point. This enabled Bernstein to demonstrate the link between the speed of propagation of the negative variation, and that of the nerve signal. The apparatus also allowed measurement of the detailed time course of the action potential, as well as the resting potential and overshoot of the action potential (Bernstein, 1868). Bernstein went on in 1896 to employ Walther Nernst’s electrolytic theory to speculate that it was K\(^+\) ion selectivity of the membrane that was responsible for the resting potential. Bernstein later went on to describe a membrane theory of excitation describing negative charges inside of the membrane and positive charges outside of the membrane with excitation resulting in a break of the barrier between the two and current flow (Bernstein, 1912).

It was Charles Ernest Overton who was able to demonstrate that Na\(^+\) ions are required for producing the negative variation and postulated that excitation resulted from exchange of Na\(^+\) and K\(^+\) ions (Overton, 1902).

It was in this setting that the concept of excitability was investigated by Weiss and then building on this by Lapicque which will be discussed in subsection 1.6.

1.2.3 Lucas, Adrian and the all or nothing law

The all or nothing nature of the nerve response was demonstrated in Cambridge through the work of Keith Lucas and subsequently his student Edgar Adrian who later became Lord Adrian. Building on a hypothesis of Francis Gotch, Lucas first showed that by increasing stimulus intensity on the nerve supplying a muscle which had been dissected down
to only a small number of fibres, one could observe discrete increments in the resulting contraction and demonstrate that excitable tissues do indeed display an all or nothing phenomenon of excitation (Lucas, 1905, 1909).

Adrian went onto to use the capillary electrometer and vacuum tube amplifier (the circuit diagram which he had acquired from Gasser) to demonstrate electrical activity in single fibres innervating muscle spindle afferents (later other fibres) and show that there was a stimulus response relationship of the frequency of these discharges but no change in amplitude; thus being the first to discover frequency encoding of the neural response (Adrian, 1926; Adrian and Zotterman, 1926). The discovery was in some ways serendipitous; initially Adrian remarked on the noise which his experimental setup was producing with his very sensitive amplification system and had sought the cause, only to realise the muscle was hanging in the preparation and when the weight was supported the “noise” would disappear. This lead to his appreciation of muscle spindle afferent single fibre discharges as the cause. In other experiments Lucas and Adrian, using local application of alcohol vapours to block conduction, also described the propagating and regenerating nature of the action potential (Adrian, 1912). Adrian went onto receive the Nobel Prize in Physiology or Medicine in 1932 in conjunction with Charles Sherrington, a man he much admired whose remarkable work on reflexes and proprioception was recognised.

1.2.4 Erlanger and Gasser use the cathode ray oscilloscope

Joseph Erlanger and Herbert Gasser in 1922 made the first very accurate recordings of the compound action potential of nerve by adapting the cathode ray oscilloscope (Braun tube) for the study of nerve (Gasser and Erlanger, 1922; Erlanger and Gasser, 1924). For the first time the action potential could be depicted in accurate time which had previously not been possible (without either mathematical or intricate mechanical correction) using the string galvanometer or the capillary electrometer which were the predominant recording instruments of the time. This work and the papers which followed detailing the compound action potential and also classifying the subtypes of fibre by speed earned Erlanger and
Gasser the joint Nobel Prize in Physiology or Medicine in 1944.

1.3 A mathematical description of the action potential and saltatory conduction

1.3.1 Hodgkin and Huxley

With accurate recording devices the problem of how the nerve action potential propagated was convincingly shown by Hodgkin (1937a,b) who used a cold block of the nerve to demonstrate that action potentials arriving at the block could influence the excitability of the axolemma distal to the block thus showing the local circuit in action (an idea originating with Hermann in 1872).

Cole and Curtis (1939) used the Wheatstone bridge and the squid giant axon preparation to demonstrate the dramatic rise in membrane conductance (with little change in capacitance) during the passage of an action potential. The authors showed that the conductance rise occurred at the inflection point of the action potential, which separated the potential change due to the local circuit from that speculated to arise from the area of now excited membrane itself and thus showed the conductance change to be intricately connected to the generation of the action potential as predicted by Bernstein’s membrane theory of excitation.

The groups of Cole and Curtis, Hodgkin, Huxley and Katz advanced the study of electrophysiology hugely using the squid giant axon and the intracellular micropipette. With this experimental setup they were able to show that there was an overshoot beyond 0mV in the intracellularly recorded action potential and that this current was carried by sodium ions (Hodgkin and Huxley, 1939; Curtis and Cole, 1942; Hodgkin and Huxley, 1945; Hodgkin and Katz, 1949).

1.3.2 The voltage clamp

Using the intracellular microelectrode and a careful addition of two wires insulated from each other, the voltage clamp technique was made possible by Kenneth Cole and George Marmont in 1947 (this is recalled by Sir Andrew Huxley (1996) in his memoir of Kenneth Cole). The voltage
clamp technique enabled elucidation of mechanisms hitherto impenetrable to analysis and has become a mainstay of electrophysiology since that time.

Voltage clamping is a technique where one wire is used to inject current and the other wire is used to measure intracellular voltage, this enables voltage to be accurately maintained within the cell via use of a feedback amplifier which varies the amount of current injected depending on the propensity of the intracellular voltage to deviate from its clamped level. The result of this is that actual ionic currents flowing through the membrane can be derived from the current required to counteract their effect.

With a series of papers in 1952 it was Alan Hodgkin and Andrew Huxley who elegantly described the ionic basis of the action potential including a mathematical analysis which would reproduce their observations (Hodgkin and Huxley, 1952b,a,c,d). It was this work that earned Hodgkin and Huxley the Nobel Prize in Physiology or Medicine in 1963 (also shared with John Eccles).

### 1.3.3 Currents underlying the action potential

In the papers for which Hodgkin and Huxley received their Nobel Prize, is elegantly laid out the evidence behind sodium and potassium ions and their voltage dependent flow being the basis for the action potential. Also laid out, is a mathematical model describing the voltage dependencies of the sodium and potassium currents. It should be noted that at no point do they refer to channels. Figure 1.2 shows the action potential as an increase in voltage compared with the resting state which reaches a maximum over the course of a millisecond before declining and overshooting the original resting voltage with more prolonged period below resting voltage. The contributing conductances to this voltage change can be seen with an initial rise in sodium conductance being driven by and then maintaining and accelerating the voltage increase (the process of activation of the sodium current) before switching off (fast inactivation). Potassium conductance can be seen activating more slowly than the sodium conductance before also turning off in a slower process lagging behind the voltage decrease and subsequent overshoot.
Figure 1.2: The action potential and conductances. The action potential labelled V in red, shows membrane voltage (V_m) in mV on the leftward y-axis plotted against time in ms on the x-axis. Overlaid on the same time scale, are the membrane conductances of sodium (g_{Na} in green), potassium (g_{K} in blue) and total (g_{tot} in purple). Conductance is plotted in mS/cm on the rightward y-axis. Description in text.

1.3.4 Saltatory conduction

In an excellent history on myelin, Boullerne (2016) summarises the important concept and discovery of saltatory conduction which was first proposed by Lillie (1925) and convincingly evidenced by Tasaki (1939). Boullerne explains how Lille used an iron wire with an insulating glass surrounding which was interrupted at intervals as a model of a myelinated nerve to demonstrate surprisingly fast conduction. After further investigation, and building evidence, including from Hodgkin providing a mathematical description of the local circuit and “electrotonic” potential, Tasaki was able to show that when 3 nodes were blocked with anaesthetic, conduction was blocked, however, when 1 or 2 nodes only were blocked the conduction was maintained. This demonstrated the local circuit in action and that a distant node could be excited provided the membrane potential had not dropped below a certain threshold.

Saltatory conduction is thus the concept that nervous transmission occurs by active regeneration of the membrane action potential at a series of discrete sites along the nerve, the nodes of Ranvier. The inter-
vening spread of potential, across the internode, occurring by passive electrotonic spread. Figure 1.3 from Burke et al. (2001), illustrates the saltatory nature of the action potential and that the sodium and potassium channels are clustered in different sections of the axon to facilitate this. The effect of this is to increase conduction velocity and also reduce metabolic demands on the axons resulting from regenerating ion gradients. Whereas the saltatory nature of conduction in myelinated fibres had been shown by 1939, it was not until the 1960s and 1970s that the idea of ion channels was formed. A summary of historical figures is given in Table 1.1.

![Image removed due to copyright](image.jpg)

**Figure 1.3:** Saltatory conduction. Sodium channels and slow potassium channels are clustered at the node of Ranvier where their density is much higher than at the internode. Fast potassium channels are concentrated in the paranodal region. This organisation together with the myelination of the internode allows for the process of saltatory conduction. Figure from Burke et al. (2001).

### 1.4 From currents to channels

Thus far, the study of nervous transmission had identified the potential changes involved and gone a long way to describing the constituent transmembrane ionic currents of the action potential. The question of what mediated these currents however had not been resolved.
Table 1.1: A table showing selected figures in the history of neurophysiology, the period in which they lived and prominent ideas or findings associated with them.

<table>
<thead>
<tr>
<th>Name</th>
<th>Period</th>
<th>Selected Ideas/Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galen</td>
<td>129-216</td>
<td>Spirits of motion</td>
<td>Galen (1549)</td>
</tr>
<tr>
<td>Descartes</td>
<td>1596-1650</td>
<td>Reflex action</td>
<td>Descartes (1662)</td>
</tr>
<tr>
<td>Swammerdam</td>
<td>1637-1680</td>
<td>Frog nerve-muscle preparation</td>
<td>Swammerdam (1758)</td>
</tr>
<tr>
<td>Galvani</td>
<td>1737-1798</td>
<td>Electrical excitation of nerve</td>
<td>Galvani (1791)</td>
</tr>
<tr>
<td>Matteucci</td>
<td>1811-1868</td>
<td>Proof of “animal electricity”</td>
<td>Matteucci (1844)</td>
</tr>
<tr>
<td>du Bois-Reymond</td>
<td>1818-1896</td>
<td>Measurement of “negative Schwankung”</td>
<td>du Bois-Reymond (1849)</td>
</tr>
<tr>
<td>von Helmholtz</td>
<td>1821-1894</td>
<td>Velocity of the nerve impulse</td>
<td>Helmholtz (1850)</td>
</tr>
<tr>
<td>Bernstein</td>
<td>1839-1917</td>
<td>Time course of the nerve impulse</td>
<td>Bernstein (1868)</td>
</tr>
<tr>
<td>Weiss</td>
<td>1859-1931</td>
<td>Weiss’ Law ( Q = a + bt )</td>
<td>Weiss (1901)</td>
</tr>
<tr>
<td>Overton</td>
<td>1865-1933</td>
<td>Na(^+) and K(^+) ions involved in excitation</td>
<td>Overton (1902)</td>
</tr>
<tr>
<td>Lapicque</td>
<td>1866-1952</td>
<td>Chronaxie and Rheobase</td>
<td>Lapicque (1909)</td>
</tr>
<tr>
<td>Lucas</td>
<td>1879-1916</td>
<td>All-or-nothing response</td>
<td>Lucas (1909)</td>
</tr>
<tr>
<td>Erlanger</td>
<td>1874-1965</td>
<td>Oscilloscope recording of action potential</td>
<td>Gasser and Erlanger (1922)</td>
</tr>
<tr>
<td>Weiss</td>
<td>1900-1984</td>
<td>Conductance change with the action potential</td>
<td>Cole and Curtis (1939)</td>
</tr>
<tr>
<td>Hodgkin</td>
<td>1914-1998</td>
<td>Mathematical description of the action potential</td>
<td>Hodgkin and Huxley (1952d)</td>
</tr>
<tr>
<td>Huxley</td>
<td>1917-2012</td>
<td>Mathematical description of the action potential</td>
<td>Hodgkin and Huxley (1952d)</td>
</tr>
<tr>
<td>Hille</td>
<td>1940-</td>
<td>Concept of ion channels</td>
<td>Hille (1970)</td>
</tr>
<tr>
<td>Sakmann</td>
<td>1942-</td>
<td>Patch clamp technique</td>
<td>Hamill et al. (1981)</td>
</tr>
<tr>
<td>Neher</td>
<td>1944-</td>
<td>Patch clamp technique</td>
<td>Hamill et al. (1981)</td>
</tr>
</tbody>
</table>

Hodgkin et al. (1952) proposed mechanisms by which the relevant currents may pass through a membrane. Mechanisms included “carrier particles” which would ferry ions across, or conversely charged activating “particles” which changed position within the membrane and allowed ions through. Although the descriptions came very close the term channel was not used.

It was Hille (1970) who formalised the concept of ion selective membrane channels being the pathways allowing the movement of ions involved in the currents of the action potential. In a review of experimental data Hille linked his thinking closely with the Hodgkin and Huxley equations stating “a well-developed mathematical model of a phenomenon contains clues as to the underlying physical processes”.

Finding and purifying a putative sodium channel protein was enabled by using specific neurotoxins known to affect the sodium current. In a re-
view of sodium channel structure and function Catterall (2012) describes his own work and that of others in identifying large $\alpha$ subunits of 260 kDa and smaller $\beta$ subunits of 30-40 kDa by photoaffinity labeling with a modified scorpion toxin. Rat brain sodium channels for example were found to have a non-covalently associated $\beta_1$ subunit and a disulfide-linked $\beta_2$ subunit (Figure 1.4).

Two technologies, the patch clamp technique and cloning of ion channels, allowed for the explosion in knowledge of the molecular structure and function of sodium, potassium, and other channels of nerve.

**Figure 1.4:** Subunit structure of the brain sodium channel depicting the $\alpha$ and $\beta$ subunits and their relationship. Subunit structure of the rat brain sodium channel. The figure shows binding sites of Scorpion toxin (ScTX) and Tetrodotoxin (TTX) which allow purification and isolation of the channel and subunits. The $\alpha$ and $\beta_2$ subunits are linked by a disulphide bond shown as S-S. Adapted from Catterall (2012)

### 1.4.1 Patch clamp technique and channel cloning

In the late 1970s and early 1980s Erwin Neher and Bert Sakmann developed the patch clamp technique (Hamill et al., 1981; Neher et al., 1978; Neher and Sakmann, 1976).

The patch clamp technique uses a polished glass pipette pushed up to a nerve membrane to generate a tight high resistance seal, this combination of pipette and membrane can then be investigated electrophysiologically with very small current and voltages measured. It has enabled
recording from single ion channels and elucidation of their properties. This work also earned Neher and Sakmann the Nobel Prize in Physiology or Medicine in 1991.

Sodium channels were the first voltage-gated ion channel to be sequenced using cDNA cloning by Noda et al. (1984) (almost coinciding with the development of patch clamping). Cloning and purification allowed demonstration that sodium channel α subunits are composed of approximately 2000 amino acid residues organised in four homologous domains, which each contains six transmembrane segments (Figure 1.5). These alpha subunits were seen to variably associate β subunits in heterodimers or trimers, these associations in the membrane have been shown to alter the voltage dependent behaviour of the channels and explain some of the diversity seen in the nerve. The sodium channel was the largest protein to have been cloned using the cDNA technique at the time.

Most recently the group of Catterall have been the first to describe the crystal structure of the bacterial sodium channel (Payandeh et al., 2011). This has provided very detailed insight into the formation of the pore, selectivity filter and mechanism of activation amongst other characteristics.

1.5 Variety of sodium channels and their expression

1.5.1 α subunits

The main determinant of sodium channel behaviour is the α subunit. The α subunits can be described by their individual characteristic electrical properties, susceptibility to agonists and antagonists and the tissue in which they are expressed. Individual axons may express more than one type of α subunit. Table 1.2 shows the 9 sodium channel α subunits (the 10th does not appear to function as a voltage gated channel). These are named according to a standard nomenclature (Goldin, 2001). Table 1.2 shows the sensitivity of each channel type to tetrodotoxin, the activation threshold, inactivation rate and tissue of distribution. A comprehensive online database and review, including of the voltage gated sodium
Figure 1.5: The primary structures of the subunits of the voltage-gated sodium channels. Figure and legend from Catterall (2012). Cylinders represent probable α-helical segments. Bold lines represent the polypeptide chains of each subunit with length approximately proportional to the number of amino acid residues in the brain sodium channel subtypes. The extracellular domains of the β1 and β2 subunits are shown as immunoglobulin-like folds. Y, sites of probable N-linked glycosylation; P in red circles, sites of demonstrated protein phosphorylation by PKA (circles) and PKC (diamonds); green, pore-lining segments; white circles, the outer (EEEE) and inner (DEKA) rings of amino residues that form the ion selectivity filter and the tetrodotoxin binding site; yellow, S4 voltage sensors; h in blue circle, inactivation particle in the inactivation gate loop; blue circles, sites implicated in forming the inactivation gate receptor. Sites of binding of α- and β-scorpion toxins and a site of interaction between α and β1 subunits are also shown. Tetrodotoxin is a specific blocker of the pore of sodium channels, whereas the α- and β-scorpion toxins block fast inactivation and enhance activation, respectively, and thereby generate persistent sodium current that causes depolarisation block of nerve conduction. Tetrodotoxin has been used as a tool to probe the pore of the sodium channel, whereas the scorpion toxins have been valuable as probes of voltage sensor function.
channel family, are maintained by The British Pharmacological Society (BPS) and the International Union of Basic and Clinical Pharmacology (IUPHAR), now developed jointly with funding from the Wellcome Trust. At the time of writing, this serves as an excellent, updated resource for further information on voltage gated sodium and other ion channels in general.

Any given mammalian nerve, whether it be myelinated or un-myelinated, will express a certain repertoire of \( \alpha \) subunits of the sodium channel along with associated \( \beta \) subunits; the channels expressed and their localisation may vary during development (Boiko et al., 2001). These channels localise to certain subcellular regions for example Na\(_\text{V}1.6\) localising to nodes of Ranvier, synapses, and dendrites in both motor and sensory fibres; the main channel being responsible for the sodium current in motor axons thought to be Na\(_\text{V}1.6\) which produces a fast inactivating, tetrodotoxin-sensitive sodium current (Caldwell et al., 2000). One major categorisation of \( \alpha \) subunits is into Tetrodotoxin sensitive and resistant, Na\(_\text{V}1.8\) and 1.9 for example being resistant. Na\(_\text{V}1.7\), 1.8 and 1.9 have a special role in pain in small and un-myelinated fibres. Sensory neurons of the DRG (dorsal root ganglion) have been shown to express Na\(_\text{V}1.1\), 1.6, 1.7, 1.8, and 1.9; recently there has been shown to be more overlap than previously thought between myelinated and un-myelinated fibres, with Na\(_\text{V}1.8\) being found in both, having previously thought to have been restricted to small diameter fibres (Ramachandra et al., 2013). Since the discovery that a mutations in SCN9A are responsible for the condition of congenital insensitivity to pain (Cox et al., 2006), sodium channel \( \alpha \) subunits are also a major interest for drugs targeted at pain (Bagal et al., 2014).

The repertoire of sodium channels an axon may express is also affected by injury, for example Na\(_\text{V}1.3\) may be up-regulated after nerve injury in dorsal root ganglion neurons and mediate neuropathic pain, despite being primarily expressed in the central nervous system. The cell adhesion molecule contactin has been shown to be involved in this process, itself also being up-regulated after injury.

Evidence from both electrophysiology and binding studies, for example with labelled saxitoxin, in myelinated axons has shown Na+ channels cluster at the nodes of Ranvier (Ritchie and Rogart, 1977). In non-myelinated axons a uniform distribution was thought to be found. More
recently there is evidence of sodium channels clustering on lipid raft in very small un-myelinated fibres possibly to enable a form of saltatory conduction (Neishabouri and Faisal, 2014).

The question of which Na\(^{+}\) channel subtypes are relevant to the nodal and internodal currents is not fully resolved. The persistent sodium current at the node, for example is possibly a behaviour of the NaV1.6 channel caused by delayed inactivation. Yan et al. (2017) used cloned Na\(^{+}\) subtypes and site-directed mutagenesis, to show that calmodulin had a strong regulating power, via the intracellular C-terminal domain, on persistent sodium current measured through channels NaV1.5, NaV1.2 and NaV1.6.

### 1.5.2 \(\beta\) subunits

There are 4 \(\beta\) subunits in mammals, \(\beta1\), \(\beta1A\), \(\beta2\) and \(\beta3\) the first 2 being a splice variant of the same gene. The corresponding genes being SCN1B, SCN2B and SCN3B. The coexpression of \(\beta\) subunits results in significant changes in the voltage dependent behaviour of channel. Sodium channel \(\beta\) subunits roles are varied and include modulation of voltage dependent behaviour, acting a cell adhesion molecules to influence cell migration, cytoskeletal interaction via ankyrin in the subcellular placement of sodium channels and mediation of sodium channel clustering, for a review see Isom (2001).

<table>
<thead>
<tr>
<th>Channel subtype</th>
<th>Gene symbol</th>
<th>Tetrodotoxin sensitivity</th>
<th>Activation threshold</th>
<th>Inactivation rate</th>
<th>Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(_{1.1})</td>
<td>SCN1A</td>
<td>Sensitive</td>
<td>Low</td>
<td>Fast</td>
<td>Mainly CNS</td>
</tr>
<tr>
<td>Na(_{1.2})</td>
<td>SCN2A</td>
<td>Sensitive</td>
<td>Low</td>
<td>Fast</td>
<td>Mainly CNS</td>
</tr>
<tr>
<td>Na(_{1.3})</td>
<td>SCN3A</td>
<td>Sensitive</td>
<td>Low</td>
<td>Fast</td>
<td>Embryonic CNS and PNS, Adult CNS, injured DRG</td>
</tr>
<tr>
<td>Na(_{1.4})</td>
<td>SCN4A</td>
<td>Sensitive</td>
<td>Low</td>
<td>Fast</td>
<td>Skeletal muscle</td>
</tr>
<tr>
<td>Na(_{1.5})</td>
<td>SCN5A</td>
<td>Resistant</td>
<td>Low</td>
<td>Medium</td>
<td>Cardiac muscle</td>
</tr>
<tr>
<td>Na(_{1.6})</td>
<td>SCN8A</td>
<td>Sensitive</td>
<td>Low</td>
<td>Fast</td>
<td>PNS and CNS</td>
</tr>
<tr>
<td>Na(_{1.7})</td>
<td>SCN9A</td>
<td>Sensitive</td>
<td>Low</td>
<td>Fast</td>
<td>Mainly DRG</td>
</tr>
<tr>
<td>Na(_{1.8})</td>
<td>SCN10A</td>
<td>Resistant</td>
<td>High</td>
<td>Slow</td>
<td>DRG</td>
</tr>
<tr>
<td>Na(_{1.9})</td>
<td>SCN11A</td>
<td>Resistant</td>
<td>Low</td>
<td>None</td>
<td>DRG</td>
</tr>
</tbody>
</table>

Table 1.2: Mammalian sodium channel \(\alpha\) subunits.
CNS - Central nervous system, DRG - Dorsal root ganglion, PNS - Peripheral nervous system
1.6 The concept of excitability

Once it was known that nerve and muscle could be stimulated electrically, the question arose as to what stimulation was necessary and sufficient to elicit a response from the tissue and how this varied. This question was first investigated by George Weiss, subsequently followed up by Louis Lapicque and leading to the technique which is used today in this thesis, namely that of testing the excitability of a whole nerve.

Electrical recording and stimulation at the beginning of the 20th century were limited in their ability to discriminate small time periods in the millisecond range (except with repeated procedures as employed by Bernstein with his ingenious differential rheotome). The result of this was a panel of electrophysiologists including Du Bois Reymond, Helmholtz, Marey, Lipmann, d’Arsonval, Joubert who charged Georges Weiss with studying how to produce devices that were mutually comparable for use by electrophysiologists.

Georges Weiss initially trained as an engineer before studying medicine and obtaining his doctorate in 1889 and becoming “professeur agrégé” at the Department of Medical Physics of the Medical Faculty in Paris, in 1911 becoming full Professor. Weiss with his engineering background came up with an ingenious solution to short defined pulse stimulation. Weiss linked a voltage distributor with two electrodes each connected with a breakable conducting thread, by using a liquid carbonic acid powered air rifle with a projectile of known speed he was able to sequentially break these threads with a resulting short constant stimulus (Figure 1.6). This setup allowed Weiss to experiment with short duration pulses and double pulses of constant current. The pulses were also monophasic in contrast to the diphasic pulses in use. Weiss postulated three important theorems for the study of nerve excitability as a result of his investigations published in his paper “Sur la possibilité de rendre comparables
entre eux les appareils servant à l’excitation électrique” (Weiss, 1901):

Theorem 1  The threshold quantity $Q$ (charge) for threshold activation can be described by the formula below, where $a$ and $b$ are the constants and the variable $t$ representing the duration of the constant stimulus:

$$Q(t) = a + bt$$  \hspace{1cm} (1.1)

This is known as Weiss’ Law and described by himself as “formula fundamental”. The constant $a$ is now known as the strength-duration time constant and $b$ as rheobase (a term borrowed from and coined by Lapicque).

Theorem 2  There is always a minimum delivered energy that depends on pulse duration.

Theorem 3  The pulse shape plays no role in electrostimulation.

Weiss’ achievement was to begin to formalise the relationship between the strength of a stimulus and its duration necessary to elicit nervous activity, or strength-duration relationship. Formula 1.1 gives two constants which may be described as characteristic of a certain strength-duration relationship.

Louis Lapicque, a French neurophysiologist who later rose to the Professorship for general physiology at the Faculty of Sciences in Paris at the Sorbonne, built on Weiss’ ideas and methods (including the ballistic method for generating brief pulses) proposing the concept of “Chronaxie” and “Rheobase” Lapicque (1909).

Definition 1  Rheobase is the strength of a stimulus current that is infinitely long and which just excites (threshold).

Definition 2  Chronaxie is the duration of threshold current that is twice rheobase.

Lapicque proposed an equivalent circuit to explain his ideas which is the forerunner of the later equivalent circuit models utilised to explain nerve excitability Lapicque (1907). Using this equivalent circuit, Figure 1.7, Lapicque mathematically derived his version of Weiss’ law to calculate the stimulus required to excite a nerve.
Figure 1.6: Weiss’ experimental setup; a) Diagram of voltage distributor linked to electrodes E. Breakage of the threads initially removes a short circuit (A-B connection) allowing current to flow, then breakage of the second thread (C-D connection) breaks the circuit and terminates the stimulus. Since the velocity of the bullet from the air rifle was known to Weiss at 130ms$^{-1}$ for every centimetre of separation between the threads (to be sequentially severed by the bullet, A-B then C-D) the stimulus duration increases by 77µS. b) Adaptation of setup in a) to allow double pulse stimulation. c) Waveforms of stimuli produced from the experimental setups. Images from Weiss (1901).

Figure 1.7: Lapicque’s equivalent circuit to explain excitation of the nerve membrane with a voltage. K a capacitor representing the membrane, $\rho$ a leak resistance, R input resistor. The threshold for excitation is reached when the capacitor K reaches voltage v. Original image from Lapicque (1907).
Theorem 4 Lapicque’s theoretical relationship of the voltage across the capacitor $v$ and the applied voltage $V$ for the equivalent circuit in Figure 1.7 where the variables are defined and $t$ is time:

$$v = V \frac{R}{R + \rho} \left(1 - e^{-\frac{t}{R + \rho}}\right)$$ \hspace{1cm} (1.2)

Theorem 5 Lapicque’s proposed relationship between threshold current $I$ for a pulse duration $t$ and it’s relation to rheobase (a current, $I_{\text{rheobase}}$) and chronaxie (a time constant, $\tau_{\text{chronaxie}}$):

$$I(t) = \frac{I_{\text{rheobase}}}{1 - e^{-t/\tau_{\text{chronaxie}}}}$$ \hspace{1cm} (1.3)

Thus both Weiss and Lapicque described differing theories to explain the strength-duration relationship. Both proposed characteristic constants to described the relationship between excitation and stimulus. Lapicque coining the terms chronaxie and rheobase. Weiss’ formula, when thought of in the language of Lapicque, maybe written in terms of constants as thus:

$$Q = I_{\text{rheobase}} (t + \tau_{sd})$$ \hspace{1cm} (1.4)

It was known at the time, that the toxin curare (now known to block nicotinic acetyl choline receptors at the neuromuscular junction) would abolish the indirect response of muscle after stimulation by the nerve. The established idea of Claud Bernard was that there was an intermediate substance between muscle and nerve which was susceptible to curare. In 1907 Lapicque proposed a hypothesis which gained general acceptance, that the transmission of the nervous impulse to the muscle was due to the similarity of their strength duration curves or “isochronism”. The chronaxie, Lapicque thought, being normally identical for muscle and nerve and any drug or disturbance causing their values to diverge by more than 2:1 causing failure of excitation. The theory of isochronism was only finally discredited in the 1930s, due to Rushton’s critique and discovery of the chemical nature of neuromuscular transmission, after holding sway for many years, indicating the dominance of Lapicque at the time (Rushton, 1933). The strength-duration concept as a property of nerve by this time had been established, this useful measure is part of the protocol used in the excitability testing in this thesis described later.
A. V. Hill at University College London in 1936 provided a detailed mathematical description of two of the important concepts in the excitability tests of today, the concept of accommodation (coined by Nernst in 1908) and the time constant of stimulation (Hill, 1936). Accommodation refers to the phenomenon of the threshold of the nerve increasing over time when a depolarising stimulus (or as described at the time a cathodal make stimulus) was applied to the nerve. Hill found that the excitability of a nerve may be described by process of two distinct time courses (see Figure 1.8), one with a short time constant $k$ which described the rate at which the voltage across the nerve decayed after a stimulus and a second time constant $\lambda$ which described the rate at which the threshold of the nerve changed in response to a perturbation of membrane voltage (accommodation). Hill argued that his time constant of the nerve for stimulation $k$ was related to Lucas’ “excitation time” and Lapicque’s “chronaxie” and that his time constant of accommodation $\lambda$ was identical to Fabre’s “constante linéaire”, and Monnier’s $\tau_2$. One of Hill’s assumptions was that accommodation would lead to the voltage change required for excitation of the nerve returning to its original level given sufficient time, evidence for this comes from very depolarised nerves and Hill himself states that “In practice, under most conditions, owing to electrotonic changes of excitability (“Pfluger’s law”), it will return to a somewhat different value: but the general effects manifest” (Hill, 1936). Evidence from nerves that are in their resting condition however do not behave in line with this assumption and along with the detailed mathematical description of the action potential by Hodgkin and Huxley later revealed the flaws in Hill’s theory. Despite this, his ideas and mathematical treatment concerning the more intricate aspects of neuronal excitability paved the way for the later progress.

Prior to Hill, Joseph Erlanger working with Edgar Blair at Washington University in St Louis first investigated the subthreshold conditioning stimuli and their influence on the threshold of nerve response by use of the constant response method, the first implementation of what today maybe called threshold tracking (Figure 1.9).

By this point the concept of excitability of the nerve and the various parameters associated with it had been described, these included its strength-duration properties (chronaxie and rheobase), the recovery cycle, and the property of accommodation. This work had been largely
Figure 1.8: Hill’s explanation of accommodation. Original figure and legend from Hill (1936).
Rise of “local potential” V and of “threshold” U, at the cathode, for constant current suddenly applied. Calculated for frog’s nerve at 4°C, ordinary “accommodation,” \( k = 1 \) msec, \( \lambda = 50 \) msec: various currents, as shown. Current 1 would be “rheobase” apart from accommodation. The actual rheobase is 1.08, and its “utilization time” is shown by an arrow. \( k \) refers to the time constant of accommodation and \( \lambda \) to the time constant of excitation proper.

Figure 1.9: Diagram summarising the irritability responses at the cathode of a long (30\( \sigma \)) subrheobasic current. The rectangular figure gives the position of the make and the break of the polarizing current. Ordinates, irritability; abscissae, time in sigmas. Original figure from Erlanger and Blair (1931). Note that \( \sigma \) confusingly refers to a millisecond as pointed out by Hill (1935).
carried out in animal fibres and only in rarely in detailed examinations of human fibres, largely due to the difficulty of controlled and sensitive recording. With the advent of the voltage clamp technique, developed by Cole and Marmont and employed by Katz, Hodgkin and Huxley, nerve conduction studies proving clinically more useful and then the patch clamp technique developed by Erwin Neher and Bert Sakmann in the 1970s (Neher et al., 1978) the focus shifted away from description of the gross excitability characteristics of myelinated nerve to either nerve conduction studies for clinical practice or voltage and patch clamp studies for underlying mechanisms.

A further concept to introduce at this stage is that of the nature of ion channel blocking agents, as these compounds will be described in the coming sections. Ion channel blocking agents were also an integral tool in the progress and discoveries described above. The concept of “frequency-” or “use-dependent” block (which are often used interchangeably) were formalised by Courtney (1975) and are a different property from that referred to as “tonic” block. A third concept of “state-dependent” block arose from Hille’s 1977 papers proposing the modulated receptor hypothesis (Hille, 1977a). It is important to note that the properties of state-dependent, use-dependent, and tonic block are not independent; and, to a greater or lesser extent, are seen in virtually all sodium channel blocking agents depending on how they are tested. These concepts will be discussed more fully in chapter 6.

1.6.1 Progress in nerve excitability

Joseph Bergmans and the re-emergence of excitability

Joseph Bergmans thesis of 1970 details and summarises his experiments performed on single motor units in human nerve (largely his own!) and their excitability properties (Bergmans, 1970). Bergmans was able to show, in man, a reliable method for recording single motor units using surface stimulation and was able to construct excitability curves including measuring strength-duration properties, chronaxie and rheobase and recovery cycle properties including the early H1 and late H2 phases of subexcitability. Experiments were performed on normal variability and the effects of ischaemia and recovery from ischaemia. These investiga-
tions showed that one could determine excitability of a motor unit using either latency of its response or the threshold for its stimulation using surface electrodes and that the two measures corresponded well. To determine the threshold of a fibre in any particular circumstance, Bergmans would manually adjust the stimulus and measure the smallest stimulation strength giving 5 consecutive responses, thus the employing the concept we shall later come across in an automated way of threshold tracking. Curves constructed from this method corresponded very well to those constructed using measurement of conduction latency.

Developmental of modern excitability testing

Professor Hugh Bostock (at University College London, Institute of Neurology, London) brought the concept of nerve excitability measured by the threshold tracking technique into the computer age. Bostock’s contribution has effectively brought the study of nerve excitability to a point where it is clinically applicable and relatively automated. He along with other authors have elegantly linked basic axonal physiology to genetic mutations and abnormalities of ion channel function through an electrical model.

Bostock et al. (1983) first sought to apply the concepts of excitability to the pathological condition of demyelination using a rat model and diphtheria toxin to induce demyelination with the technique of threshold tracking. They showed that the strength duration curves measured in-vivo fitted Weiss’ formula remarkably well and the formula of Lapicque less well (see section 1.6). The authors then went onto demonstrate that, in the case of paranodal demyelination, the strength duration time constant (SDTC) at affected nodes of Ranvier showed a large increase compared with normal nodes, but the rheobase was relatively unchanged. This was an elegant demonstration linking a pathology to changes in electrical excitability parameters.

Bostock (1983) went onto describe these results in a mathematical way using models which built upon the original Hodgkin and Huxley work of 1952 using the model of myelinated nerve proposed by Goldman and Albus (1968). Again it was shown that the modelled charge duration plot (QT) was best matched by Weiss’s formula. The main insight was to describe how SDTC was likely to be affected by electronic time constant of
the stimulated membrane along with the rate of sodium channel activation, and the rheobase by variation in conductances of the membrane. The case of demyelination prolonging the SDTC was postulated mathematically as being due to an increase in area of the node, the membrane of which had a high capacitance with relatively little conductance.

This mathematical treatment explaining electrophysiological results seen in myelinated nerve opened up the possibility of gaining insight into disease and normal function of nerves in both animal and human by this form of excitability testing.

Barrett and Barrett (1982) published a paper which provided an explanation for a hitherto mysterious phenomenon, the depolarising after potential. Based on microelectrode recordings from lizard and frog nerve, the authors describe the depolarising after potential as resulting from a discharge from the internode to the node of a charge that had built up on the internodal membrane as a result of the action potential which had not been able to dissipate due to the long electronic time constant of the internodal membrane compared to the node and the large resistance of the myelin. This charge was therefore postulated to flow back to the node via the paranode resulting in the depolarising after potential and accounting for the superexcitability seen in the recovery cycle. The addition of a submyelin pathway between the internode and the node to the equivalent circuit would prove very valuable and inform future mathematical models used in nerve excitability testing.

Baker et al. (1987) described in detail the ionic currents responsible for the elements of excitability seen to vary as a result of electrotonus, namely the fast and slow potassium conductances located mainly in the internode and node respectively and the hyperpolarising activated inward rectifying conductance (Ih). They also described the contribution of these conductances to the recovery cycle and including the H1 and H2 subexcitability described by Joseph Bergmans.

Bostock and Baker (1988) went on to describe this technique of "threshold electrotonus" applied to humans and demonstrate evidence for the 2 types of potassium channel present in human nerve by analogy with the animal data. The authors presented an equivalent circuit which may explain this accommodative property. This model is developed over time by Hugh Bostock and a fuller version is published by Bostock et al. (1991) which largely built upon the findings of Barrett and Barrett (1982),

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this electrical model is shown in Figure 1.10.

Figure 1.10: Figure 2 and adapted legend from Bostock et al. (1991). Equivalent circuit used to model behaviour of human motor axons. Outside represents the peri-axonal space and inside the intra-axonal space. Model comprises single node and internode, equivalent to a space-clamped fibre. $C_n$, nodal capacitance; $C_i$, internal capacitance; $C_m$, capacitance of myelin sheath; $R_{il}$, internal leak resistance; $I_{pump}$, electrogenic $Na^+$-$K^+$ pump; $Na$, $K_f$, and $K_s$ are the sodium, fast and slow potassium currents; $L_k$ a leak current.

Bostock and Rothwell (1997) made a further step in describing the conductances responsible for axonal excitability in myelinated fibres by ascribing the longer SDTC and lower rheobase found in human sensory fibres when compared with motor fibres to a higher proportion of persistent sodium conductance present at the node. These data were again analysed by use of a mathematical model.

The parameters on which the mathematical model is based have largely come from in-vivo and ex-vivo recording of rat and human nerve. For example the Na$^+$ current parameters used are from current and voltage clamp recordings of single human myelinated nerve fibres by Schwarz et al. (1995). Relevant to this thesis, is the adaptation of a technique for recording a dissected rat saphenous nerve which is still innervating the skin, known as the skin-nerve technique. This technique applies nerve excitability profiling to an ex-vivo preparation and allows application of experimental compounds in order to assess their effect on
nerve excitability. The technique is described by Maurer et al. (2007), and is further described in methods section 3.1.

**Nerve excitability profile (NEP)**

The cumulative work on mathematical models and automating nerve excitability testing using computers, as well as investigation into a number of human diseases, led to the formalisation of the technique of automated threshold tracking. A custom built software programme called QTRAC (copyright Institute of Neurology London) and a protocol developed during a meeting in Trondheim Norway, called the TROND protocol, is described by Kiernan et al. (2000). The TROND protocol of consists of a stimulus-response curve, a strength(or charge)-duration curve, threshold electrotonus, the recovery cycle and an IV curve (or current-threshold curve), these will be described further in methods section 3.3.

The output of this protocol generates a host of standard measurable parameters which we shall refer to as the nerve excitability profile or NEP for the remainder of this thesis.

### 1.7 A brief history of sodium channel inhibiting compounds

#### 1.7.1 Local anaesthesia

Cocaine derived from the coca leaf brought about the advent of modern day local anaesthesia. The coca leaves of the shrub *Erythroxylum coca* were widely used in the Americas and there, common use was first documented by Spanish settlers in the 16th Century. The first reference to the anaesthetic effects of coca was by the Spanish Jesuit Bernabé Cobo (1582-1657) referring to alleviation of toothache in 1653. The active principle of the coca leaf was isolated by Albert Niemann (1834-1861) in 1860 and named cocaine. The structure was not fully elucidated until 1898 by Richard Willstätter, who went on with colleagues in Munich and the Merck Laboratory in Darmstadt to synthesise artificial cocaine in 1923. It was at the University of Würzburg where Basil Von Anrep in 1880 after experiments on animals and himself recommended cocaine
as a surgical anaesthetic.

At about the same time Austrian physician Sigmund Freud (1856-1939) was experimenting with cocaine and interested in its stimulant effects. Freud encouraged Viennese ophthalmologist Carl Koller (1857-1944) to perform, on 11 September 1884, the first human operation using cocaine as a local anesthetic on a patient with glaucoma. Koller’s paper on the subject was translated and published in the Lancet later in that year sparking international interest and arguably the birth of modern day local anesthesia (Koller, 1884).

As use became more widespread the dangers of addiction and deaths associated with overdose came to the fore and safer alternatives were sought. The development came with a para-aminobenzoic derivative of cocaine, novocaine, developed and patented by German chemist Alfred Einhorn (1856-1917) in Hessen Germany in 1905. Novocaine (rechristened procaine in the United States) was found to be effective and safe, save some allergic reactions and needing co-administration with adrenaline. After this it was not until 1943-1946 that a distinctly improved compound was developed (chemically very different from novocaine) and that was the xylidine derivative lidocaine by Nils Löfgren and Bengt Lundquist. Following this came mepivacaine and bupivacaine in 1957, prilocaine in 1969, etidocaine, articaine in 1972, ropivacaine in 1983 and more recently liposomal bupivacaine in long acting formulations. For a fuller history of cocaine see “The coca leaf and cocaine a history” (Calatayud and González, 2003).

1.7.2 Anticonvulsants

The relevance of epilepsy and anticonvulsants to voltage gated sodium channels is that many of the drugs used to treat this disorder today and historically have had their primary mode of action on voltage gated sodium channels. They are also common medications used to treat neuropathic pain. Today there is an increasing recognition of the link between genetic abnormalities of the sodium channel and epilepsy syndromes. A very good history of pharmaceutical develop of anticonvulsants can be found in the review by Shorvon (2009).

Early work on the treatment of epilepsy centred on the belief that there was an intimate link between the sedative action of a drug and its
anticonvulsant properties. Potassium bromide was the first drug which seems to have been effective against seizures and reinforced this belief. Phenobarbital was the next drug to prove effective in the condition developed from barbituric acid in 1912 and marketed under the name Luminal. Phenobarbital was also thought to be effective in epilepsy due to its sedative nature. Prior to modern anticonvulsants initially bromides and then, by 1930, phenobarbital (and a few related compounds) provided the mainstay of pharmacological treatment of epilepsy (phenobarbital is still very widely used today for certain indications). A drug similar to the later phenytoin, phenylethylhydantoin or Nirvanol was used as a hypnotic and found to be effective in epilepsy by 1931 although it had problems with toxicity.

Putnam and Merritt’s investigations of 1937-45 heralded a new era of rational drug discovery (in the context of epilepsy) which produced the drug phenytoin alternatively known as diphenylhydantoin (Merritt and Brenner, 1947; Merritt and Putnam, 1940, 1939, 1938a,b). These investigators’ contributions were multifold. Firstly they improved the technique for electrical induction of seizures and developed the idea of electrical seizure threshold in animals. Secondly they were the first to employ large scale screening of potential therapeutic compounds in a systematic way and rating them in terms of anticonvulsant activity. Thirdly they broke the contemporary idea that sedation was a necessary property for anticonvulsant activity. Fourthly the idea of screening compounds with a common organic chemical structure, in this case the phenyl radical. Finally they showed that drugs could have differential effectiveness against different seizure types. Out of these many investigations in animal, one of the first and most effective compounds in the treatment of epilepsy was phenytoin. The success of this approach and their contribution is highlighted by rapid increase in the number of clinically available drugs for the treatment of epilepsy their approach stimulated, summarised in Figure 1.11. Although phenytoin had been recognised as a “membrane stabiliser” and anti-arrhythmic in the 1950s it was not until 1972 when its action on neuronal voltage gated sodium channels was described more fully on the squid giant axon using the voltage-clamp technique by Lipicky et al. (1972).

Carbamazepine was discovered in 1953 by Walter Schindler at J.R. in Basel, Switzerland (Schindler and Häfliger, 1954). After trials in animals
showed promising anticonvulsant effects, Bonduelle and Lorge separately in 1962 were the first to report successful use of carbamazepine in humans (Blom, 1962; Ger, 1964). Due to its clinical similarity to phenytoin, carbamazepine was tested using voltage clamp in the marine tube worm axons using voltage clamp in 1974 and it’s action on voltage gated sodium channels demonstrated (Schauf et al., 1974).

Figure 1.11: Cumulative increase in antiepileptic drugs available to the medical community. Horizontal axis: year of introduction. Vertical axis: number of drugs available. Table indicates key for drugs plotted. Adapted from Figure 4 Friedlander (1986).

1.7.3 Animal toxins

Due to the global importance of nervous conduction mediated by sodium channels for the survival of animals, naturally occurring toxins targeting the sodium channel have evolved as an effective defence or method of trapping prey for small animals. Some of these toxins act by inhibiting the sodium current resulting in hypoexcitability whereas others prolong the current resulting in hyper excitability or depolarising block. Scientific study and use of these toxins as tools and more recently as potential therapeutic agents has aided discovery and sub classification of sodium
channel types and their role in living organisms. Animals employing such toxins include fish, newts, scorpions, spiders, snails and molluscs. What follows is a brief account of these major classes of toxin which inhibit the channel and their discovery.

Tetrodotoxin is the most well known nerve toxin, it a small low molecular weight molecule with a unique cage structure. The effects of tetrodotoxin have a long folk history in China and Japan where the pufferfish, from which it is derived, thrive and are consumed as the delicacy fugu and only prepared by experienced chefs who know which parts of the fish to remove. Captain Cook’s crew on their round the world voyage experienced the effects of tetrodotoxin by eating the fish *Tetrodon ocellatus* and *Sparus pagrus*, these events are recorded by J.R Forster in 1778 (Forster et al., 1778). The first western language scientific study entitled “Sur les poisons toxiques du Japon” was published in 1883 (Remy, 1883). Tetrodotoxin is found in some aquatic animals including the pufferfish, the Californian newt, the blue-ringed octopus and xanthid crabs. It is thought that rather than the animals themselves producing this toxin tetrodotoxin, it is instead produced by bacteria living symbiotically within these animals and provides a defence against predators. There remains debate on this issue however with some arguing that some animals themselves produce the toxin (e.g. newts) (Lehman et al., 2004). Bacterial species found to be able to produce this toxin include *Vibrio, Aeromonas* and *Alteromonas*. It was only in the 1950s when the Japanese scientist Yokoo isolated a crude crystalline toxin, originally named Spheroidine and later renamed Tetrodotoxin, from the ovaries of puffer fish that their toxicity was fully understood. In 1964 voltage clamp techniques were applied to lobster giant axons using the sucrose gap technique. Tetrodotoxin was found to inhibit sodium currents in isolation from potassium currents and tetrodotoxin was confirmed as a sodium channel blocker with properties similar but not identical to cocaine (in terms of sodium channel effects at least) and procaine (Narahashi et al., 1964).

Saxitoxin another small molecule with a different structure to tetrodotoxin, it is the cause of “paralytic shellfish poisoning”, which is distinguished from the common gastrointestinal illness often associated with bacterial infection resulting from shellfish ingestion. Saxitoxin causes a severe neurological with symptoms including paraesthesia in the fingers, hands, and mouth, limb weakness, vomiting, and a sensation of floating. The
symptoms are very similar to those produced by tetrodotoxin. It is a well known hazard in the USA and Canada, but outbreaks have occurred in the UK (British Medical Journal, 1970). The link between the dinoflagellate *Gonyaulax catenella*, the shellfish and the illness was suspected by Meyer, Sommers, and Schoenholt after a severe outbreak in San Francisco in 1927, when they produced a historic and classical account of "muscle poisoning" (Meyer et al., 1928). A large scale study was initiated and during a further outbreak in 1932 and the link was made (Sommer et al., 1937). The illness is primarily associated with eating bivalve molluscs e.g. mussels, clams, oysters and scallops, which feed by filtering and may accumulate toxins from their environment. The toxin causing the illness, Saxitoxin, is produced by (primarily) marine dinoflagellates which are bloom-forming microalgae and filtered and concentrated in the molluscs. These marine blooms indicate hazardous times for eating shellfish "the red tide" or "luminescence of the waves". Isolation and purification of the toxin also proved a challenge and is well summarised by Schantz (Schantz, 2006). Saxitoxins is also an umbrella term for saxitoxin and its at least 21 derivatives, production of which has been shown by multiple genera of dinoflagellates and also by cyanobacteria (Deeds et al., 2008). Hille in 1968 showed that in isolated frog nodes of ranvier using voltage clamp techniques, Saxitoxin blocked sodium channels in the same way as tetrodotoxin (Hille, 1968). Saxitoxin was found to bind to the same site in nerve as tetrodotoxin, indicating the site of action of these two toxins was the same (Henderson et al., 1973).

Conotoxins originate from the marine cone snail of the genus *Conus*, and are peptides of 10-30 amino acids in length often incorporating one or more disulphide bonds. The behaviour of the cone snail was documented first by Alan Kohn and co-workers in Australia where he described the use of a "detachable, dartlike radula tooth" to inject their fish prey with a powerful neurotoxin (Kohn, 1956). The venom was sequenced and described in detail by Baldomero Olivera, Lourdes Cruz and co-workers in Utah who amongst other achievements managed the remarkable job of milking these highly venomous snails (Olivera and Cruz, 2001). During the 1980s the wide variety of pharmacological actions of these slightly varying toxins were elucidated but there remains much to be discovered and certain compounds have been of interest to the pharmaceutical industry for therapeutic use. These authors esti-
mate as many as 50,000 different conotoxins in the genus Conus. Actions are various both behaviourally and pharmacologically, they have been considered under separate “cabals” or groups with unifying aims such as a “motor cabal” which abolishes neuromuscular transmission and a “lightening-strike” cabal aimed at causing immediate immobilisation through its action on nerve directly. Targets of these toxins include channel and receptor types other than sodium channels for example potassium channels, calcium channels and the nicotinic acetylcholine receptor. Some conotoxins specifically target the sodium channel. \(\mu\)-Conotoxins are sodium channel pore blockers and are sub-type selective, the \(\delta\)-conotoxins slow or inhibit inactivation (resulting in hyperexcitability), and the \(\mu\)-O-conotoxins block the Na\(^+\) conductance independent of the tetrodotoxin binding site (mechanism unknown) (Safo et al., 2000; Ekberg et al., 2008). These toxins provide a further pharmacological tool in the armament to separate out the contribution of sodium channel subtypes and mechanisms to the action potential of certain cells.

The toxins may thus be sub classified (as well as by their NaV1.x subtype selectivity, chemical structure and organism of origin) by their site of action on voltage gated sodium channels where there are at least 7 toxin binding sites and are summarised in Figure 1.12 (Klint et al., 2012). Tetrodotoxin and saxitoxin as well as the \(\mu\)-conotoxins directly effect the pore at site 1 whereas others affecting the gating properties affect other sites.

Spider toxins represent perhaps the most numerous and diverse class of animal toxins and it is only in the last decade that the true variety has been recognised with estimates of upwards of 10million bioactive peptides being present in their venom as a collective group. From this incredible, and as yet only very partially investigated number, the spider toxins acting upon voltage gated sodium channels have been split into 12 families and all are gating modifiers of the channel rather than pore occluders (Klint et al., 2012). Certain spider venom toxins of Family 3 according to Klint may inhibit the sodium current by binding to the voltage sensor region (S3–S4 linker) of channel domain II and modifying gating (i.e., neurotoxin receptor site 4 in Figure 1.12, thus impeding channel activation and causing the channels to remain in a resting state. The tarantula toxin ProTx-II, a member of this family, has been found to be a very specific inhibitor of NaV1.7 an interesting target for analgesia
Other toxins not described more fully here found in animals (and a plant) may act as gating inhibitors or activators which results in increased sodium current during the action potential and hyperexcitability or depolarising block. Toxins in this class are found among the conotoxins but also from the sea anemone (Oliveira et al., 2004), the scorpion (Gordon et al., 1998; de Vega and Possani, 2005), the frog (Linford et al., 1998; Bosmans et al., 2004), spiders (Klint et al., 2012), and the Lily of the genus *Veratrum* which produces veratridine (Farrag et al., 2008).

### 1.7.4 Electrophysiology of sodium channel active drugs, in particular carbamazepine and phenytoin.

After the rapid rise in number of anticonvulsant drugs available largely due to the work of Merritt and Putnam, the first electrophysiology of these drugs began to be elucidated with the now available Hodgkin and Huxley model as well as the more advanced techniques of voltage clamp in the squid axon and also the patch clamp. Phenytoin and carbamazepine were among the first for the ionic effects to be described. To put the concentrations below in context, current accepted therapeutic ranges for total plasma concentration of phenytoin and carbamazepine when used in humans are 40-80\(\mu\text{mol/L}\) and 20-50\(\mu\text{mol/L}\) respectively for free plasma concentration (taking into account protein binding) these values are approximately 4-8\(\mu\text{mol/L}\) and 5-12.5\(\mu\text{mol/L}\) respectively for the free drug.

The first observation that an anaesthetic compound acted upon the sodium current was reported in 1959 by Taylor (1959) who showed the sodium current reversibly inhibited by procaine in the squid giant axon. It was not until the 1970’s that a fuller explanation of the action of sodium channels was more fully described.

Lipicky et al. (1972) examined the effects of phenytoin (5-50 \(\mu\text{mol/L}\) free concentration) on squid giant axon, and found reversible dose dependent decrease in the early transient sodium current with no change in time constant (time to peak current) and a small fixed mild effect on the K\(^+\) current. Schauf et al. (1974) first reported the effect of carbamazepine on the unmyelinated giant axons of the Myxicola tubeworm showing 500\(\mu\text{mol/L}\) carbamazepine reduced both sodium and potassium conductances by 50% and 40% respectively. These effects developing
Figure 1.12: Molecular architecture and pharmacology of NaV channels. The pore-forming a subunit of NaV channels is comprised of four homologous domains denoted I-IV connected by intracellular linkers. Each of these domains contains six transmembrane (TM) helical segments (labelled 1-6) joined by intra- or extracellular loops. TM segments 5 and 6 from each domain, along with the intervening membrane re-entrant loops (highlighted with a light green box), come together to form the channel pore and the ion-selectivity filter at the extracellular end of the pore. The highly positively charged TM segment 4 is primarily responsible for sensing changes in membrane polarization. Sodium channel inactivation is mediated by a short stretch of hydrophobic residues (the “inactivation gate”; orange balls) in the intracellular linker connecting domains III and IV. Coloured regions represent neurotoxin receptor sites. The grey circles represent the outer (EEDD) and inner (DEKA) rings of amino acid residues that form the ion-selectivity filter and constitute the proposed neurotoxin receptor site 1 for the water-soluble guanidinium toxins tetrodotoxin and saxitoxin. Some µ-conotoxin binding sites overlap with those of tetrodotoxin and are omitted for clarity. Figure and legend adapted from (Klint et al., 2012).
with the same time course, being reversible and dose dependent (ex-
perimented with 300µmol/L to 1000µmol/L much higher than therapeutic
range in humans). It was also found that the leakage current for hyper-
polarising steps was reduced and the membrane was reversibly depo-
larised.

Schwarz and Vogel (1977) studied myelinated Xenopus nerve with
phenytoin using voltage clamp technique of Dodge and Frankenhaeuser
(1958) and air-gap method of Nonner (1969). It was found that pheny-
toin (80µmol/L) hyperpolarised the membrane, elevated the threshold,
and reduced the peak of the action potential as well as reduced conduction
velocity. On voltage clamp of a node they found phenytoin 80µmol/L
reduced Na current to 45% of control, reversal potential was unchanged.
K⁺ current was reduced to 86% of control and leakage current “almost
unchanged”. Phenytoin hyperpolarised the steady state inactivation curve
by 19.5mV however the authors were unsure of the mechanism.

Hille (1977a) proposed the modulated receptor hypothesis (which will
be discussed further later in this thesis) to describe the voltage and fre-
quency dependent actions of local anaesthetic drugs. This hypothesis
provided an explanation for, and framework to further investigate many of
the actions of the local anaesthetics and anticonvulsants. The modulated
receptor hypothesis remains to this day an invaluable idea in describing
drug action.

Perry et al. (1978) elucidated the mechanism of phenytoin more thor-
oughly; the suggested mechanism up until then had been stimulation of
the sodium potassium pump, inhibition of passive sodium influx in stim-
ulated but not resting cells, reduction of calcium influx, and specific in-
terference with synaptic transmitter movements. Experiments in isolated
squid axons reported by the authors showed phenytoin had no effect
on sodium efflux (inhibited by ouabain), binding or release of ouabain
or digitoxin and a few others, thus excluding the sodium pump as the
site of action. It was also shown via microinjection of K⁺ that phenytoin
and tetrodotoxin had the same and not additive effects, to hyperpolarise
the membrane, and both reduced K⁺ efflux in the same way despite no
activity of the Na K pump (which was poisoned by ouabain). When ver-
atridine was used to open sodium channels, phenytoin and tetrodotoxin
both antagonised this action to the same degree. It was concluded that
phenytoin, like tetrodotoxin, blocked resting and excitable sodium chan-
nels and linked to the concept of it “stabilising” the membrane.

Kendig et al. (1979) examined frequency dependent block and voltage dependent conduction block in myelinated bullfrog nerve under voltage clamp. The authors discuss the possible mechanisms of frequency dependent block which phenytoin was found to produce including the possibility of the molecule trapping the channel in the inactivated state.

Courtney and Etter (1983) examined the effects of lidocaine, phenytoin, diazepam, phensuximide, phenobarbital, ethotoin, carbamazepine, and hexobarbital in single myelinated bullfrog (Rana catesbiana) nerve and muscle fibres. The authors found all drugs suppressed Na\(^+\) current in both skeletal muscles and nerve and that hyperpolarising pre-pulse removed drug effect. Local anaesthetics have channel blocking potency highly correlated with lipid distribution coefficient (Log Q). The authors also found the myelinated nerve to be about seven times more sensitive to carbamazepine than the previously reported Myxicola giant axons and that carbamazepine produced some of the highest frequency dependent block. It was shown that lidocaine, phenobarbital, phenytoin and carbamazepine all produced frequency and voltage dependent block in myelinated nerve. These authors also went onto suggest drugs may act selectively on the inactivated form of the sodium channel.

Willow et al. (1985) tested the effect of carbamazepine and phenytoin in whole cell path clamped neuroblastoma cells. Carbamazepine and phenytoin were found to hyperpolarise the steady state inactivation curve not to affect the voltage dependence of activation of the sodium channel but did reduce the peak current and . Half maximal inhibition was reported at 30\(\mu\)mol/L for each drug with inhibition increased by depolarisation and frequency. An adaptation of the modulated receptor hypothesis was provided to explain the effects seen by preferential binding of carbamazepine and phenytoin to the inactivated state of the sodium channel thus stabilising this state, with a time course of the drug unbinding to explain the frequency effects.

Advancement of voltage protocols in patch clamp allowed investigation of the channel state dynamics to be more formally assessed. These protocols induce inactivated states by means of long depolarising pulses then release this state by means of a hyperpolarising step before application of a test pulse to look for the proportion of remaining sodium current which maybe elicited. It is possible to vary the lengths of either
the depolarising step or the hyper polarising step to quantify the time courses of inactivation and recovery from inactivation.

Using these procedures Kuo et al. (1997) investigated the effects of carbamazepine and phenytoin on the sodium channel in rat CA1 hippocampal slices under patch clamp. At a hyperpolarised holding potential carbamazepine up to 100 $\mu$mol/L had little effect (no more than 10%), however when preceded by a holding potential of -70mV the same concentration abolished in excess of 60% of the sodium current. This effects was dose and voltage dependent (holding potential) consistent with the idea of carbamazepine binding preferentially to the inactivated state.

A second paradigm whereby from a hyperpolarised holding potential, a 9 second depolarising pulse preceded the test pulse allowed assessment of a shift in the voltage at which half of the channels are in the inactivated state caused by varying drug concentration. This showed that carbamazepine and phenytoin bind to the inactivated state of the sodium channel, carbamazepine with a lower affinity than phenytoin; apparent dissociation constants of $\sim 25 \mu$mol/L and $\sim 7 \mu$mol/L respectively. These compare with figures approaching 1000 $\mu$mol/L for affinity for the resting state for both drugs.

A third paradigm whereby a variable length depolarising pre-pulse was followed by a strong brief hyperpolarisation (to attempt to relieve a degree of fast inactivation, specifically from the unbound channels) lead to the calculation of a faster binding rate for carbamazepine than phenytoin ($\sim 38,000 \ M^{-1}/\sec$ and $\sim 7,700 \ M^{-1}/\sec$ respectively). This treatment thus provides an explanation for the frequency dependent characteristics seen with these drugs whereby repeated cycling through high affinity states within a certain time window may lead to an accumulation of drug bound non-conducting channels.

Errington et al. (2008) has confirmed the above findings that carbamazepine and DHP both bind to the fast inactivated state of the sodium channel, and extended them in comparison with the newer compound lacosamide. By modifying voltage protocol in cultured cortical neurons under whole cell patch clamp, the authors showed a novel mechanism of action for lacosamide having preferential affinity for the slow inactivated state of the sodium channel. It was also shown for phenytoin and carbamazepine that they did not affect the slow inactivated state. Carbamazepine and phenytoin were both also found to exhibit frequency
dependent block at 10Hz (at concentrations of 100 µmol/L).

As will be discussed in later sections (Chapter 6) a more recent and structured approach to formalising quantification of parameters devised by Lenkey et al. (2010) raise interesting questions about the terminology and conclusions drawn from voltage protocols such as those used above.

1.8 Conclusion and summary of the following chapters

This chapter has outlined the historical context in which nerve excitability testing has developed. The gradual evolution in cellular electrophysiology, drug and molecule discovery, understanding how sodium channels interact with drugs and advances in genetic understanding of disease, are at a stage where a technique applicable to humans which enables a glimpse into the in-vivo function of drugs on the ion currents underlying the action potential would provide a valuable tool. Applications potentially include the drug development industry, who are in search of novel compounds acting on the sodium channel and particular sub-types, clinicians who are in search of tools for therapeutic monitoring of drug effect in their patients and researchers for whom human volunteers and patients have often been rather inaccessible to detailed electrophysiology in comparison with the in-vitro environment. Current progress in the development of sodium channel blockers and in particular those targeting NaV1.7 and NaV1.8 including early clinical trials is reviewed in Bagal et al. (2014); Yekkirala et al. (2017). It should be noted again at this point that this technique examines the large myelinated fibres, with NaV1.6 likely being the most relevant channel. This thesis draws no conclusion or comment on which channel subtypes contribute to the currents studied. For the purpose of this thesis, a Na⁺ current is modelled which, for the sake of simplicity of the modelling, is imagined as being due to a homogenous sodium channel type.

Chapter 2 provides a continuation from the historical background given in this chapter with details of the rationale for the current experiments in the context of contemporary research findings. Chapter 2 then states the hypotheses to be tested in this thesis.

As we have seen in the historical context, voltage protocols have
been adapted over the years to address specific questions; the same is true of this thesis. Chapter 2 goes onto explain the reasoning for an adaptation of the current nerve excitability testing protocol specifically to look at use-dependent properties, using the technique of threshold tracking.

The modulated receptor hypothesis of Hille is introduced, along with the concept of sub-excitable periods after trains of impulses. Joseph Bergmans described the physiological effects of trains of impulses in myelinated nerve, noting the early sub-excitability or H1 period, mediated by slow potassium channels, and the late sub-excitability or H2 period, mediated by the sodium potassium pump.

A limitation of the current threshold tracking protocol, that it does not incorporate trains of pulses, is the reason behind designing a new protocol to threshold track at variable frequency stimulation. This it is hoped would draw out the use- or frequency-dependent properties of the drugs tested. When considering the results, both modulated receptor hypothesis and the H1 and H2 periods become very relevant.

Chapter 3 explains the methods by which the stated hypotheses will be tested. It explains how the threshold tracking method, developed by Joseph Bergmans and later Hugh Bostock, has been implemented in the context of a clinical trial of healthy human subjects taking a dose of carbamazepine. This is allied with description of the method in which the same technique can be applied to a dissected rat saphenous nerve in an ex-vivo environment. The method of adapting the current nerve excitability testing software to allow for testing use dependence are then detailed.

Chapters 4 to 6 present the results of these experiments, firstly for healthy human subjects who volunteered to take a dose of carbamazepine. Secondly for rodent nerve in an ex-vivo environment. Thirdly for human and rodent nerve under different frequency stimulation, and for the rodent nerve with the effects of carbamazepine, lidocaine and tetrodotoxin.

The results of these experiments are then discussed in 7. We shall see how explanation of the complex changes seen are not immediately susceptible to straight forward explanation.

For this reason Chapter 8 then proposes a mathematical model, which is an extension of that devised by Hugh Bostock, in order to provide an improved explanation of the findings presented in the preceding chap-
ters. The mathematical model and its adaptation in a sense are part of the conclusion in that it is an attempt to draw together all the experimental data and make sense of it.

Mathematical modelling has shown success in explaining the changes in the nerve in conditions such as renal failure and diabetes, and also with certain genetic abnormalities such as the fast potassium channel mutation causing episodic ataxia. In cellular electrophysiology, drug effects have been successfully explained with adaptations of the classical Hodgkin and Huxley model, such as in the work done by Karoly et al. (2010) on a wide variety of drugs acting on sodium channels. This provides a rationale to combine the two approaches and adapts the current Bostock model.

Chapter 9 concludes by summarising the results, what conclusions may be drawn and whether the hypothesis may be rejected or accepted. How these findings may be relevant to clinical situations and future work is discussed.
Chapter 2

Background and Hypotheses

This chapter provides a continuation from the historical background with an explanation of the rationale for the current experiments in the context of contemporary research findings. The hypotheses to be tested are then stated.

As we have seen in the historical context, voltage protocols have been adapted over the years to address specific questions; the same is true of this thesis. When Hille was formulating the modified receptor hypothesis it was on the basis of results of work on varied frequency stimulation. This chapter goes onto explain the reasoning for an adaptation of the current nerve excitability testing protocol specifically to look at use-dependent properties using the technique of threshold tracking.

2.1 Rationale for utilising the NEP to investigate carbamazepine in myelinated nerve.

There is a need for novel target engagement biomarkers to assist with development of new drugs, in particular those with effects on Na$^+$ channels (Frank and Hargreaves, 2003; Chizh and Sang, 2009). Demonstration that the NEP allows accurate detection of target engagement in healthy human volunteers would serve as a proof of principle for its role as a biomarker on those subtypes of Na$^+$ channel expressed in large myelinated nerve. The ability to perform the NEP in animal and human, in-vivo and ex-vivo settings potentially enables the NEP to provide a translational measure.
It has been described in section 1.6.1 how the technique of nerve excitability testing with through the method of threshold tracking has been developed, and that through generation of an NEP, is able to give insight into specific ion channel function, both in human and animal nerve, in-vivo and ex-vivo.

Recent work has shown that the NEP is able to detect the effects of the high affinity Na\(^+\) channel pore blocker tetrodotoxin on human nerve in a group of subjects who were accidentally poisoned by a fish soup (but survived!). Kiernan et al. (2005a) demonstrate that the effects of tetrodotoxin, maybe detected and mathematically modelled by reduction in Na\(^+\) conductance.

Tomlinson et al. (2010) has shown that the NEP is able to detect effects in peripheral nerve of the Kv1.1(KCNA1) fast potassium channel mutations in patients with episodic ataxia type 1. Fitting of this data against the mathematical model of myelinated nerve indicated a reduction in fast potassium conductance as explaining the deviation from controls. This is an example of a genetic ion channel defect being detected and its effect predicted by electrophysiology.

Isose et al. (2010) and Kuwabara et al. (2005) have assessed the Na\(^+\) channel blocker Mexiletine in patients with neuropathic pain and severe muscle cramping. These authors did find changes in SDTC, rheobase and refractoriness but not in others markers of excitability, the findings were explained as resulting from a decrease in persistent Na\(^+\) conductance and possibly in transient Na\(^+\) conductances. As alluded to in section 1.6.1, the subtypes of Na\(^+\) channels responsible for the nodal persistent sodium current remains uncertain. One must note that the NEP reflects nodal and internodal currents while being agnostic of the precise channel subtypes involved. The mathematical model itself models only a single type of transient Na\(^+\) and persistent Na\(^+\) current which is based on experimental recordings of human and rat nerve (Schwarz et al., 1995).

Development of an ex-vivo rat saphenous nerve model for performing the NEP (Maurer et al., 2007) has extended the possibility of investigating drug effects in rat models. Pilot data in this model has shown that the NEP is sensitive to the effects of carbamazepine at doses relevant to the therapeutic range in humans. Shields and Koltzenburg (2010) have presented, in poster form, data showing that concentrations as low as 3\(\mu\)mol/L of carbamazepine are detectable with the NEP. Figure 2.1
shows the results of pilot data recording the NEP in skin nerve model and administering carbamazepine, the charge duration plot Figure 2.1 a) shows an increase in rheobase (gradient of the line) and a decrease in the SDTC (the x intercept of the line). Figure 2.1 b) shows the effect on depolarising threshold electrotonus, where a reduction in excitability can be seen in response to prolonged sub-threshold depolarisation (S2 accommodation) as a result of carbamazepine.

The aim of this present study is to determine whether the effects of a Na\(^{+}\) channel blocking agent maybe detected, in healthy volunteers, by the NEP; and provide proof of principle for its role as a translational mechanism of action biomarker. Carbamazepine was chosen due to its long standing clinical use as an anticonvulsant and for its action in painful conditions; its well known safety profile and tolerability; and its well described mechanism of action of stabilising the fast inactivated state of the Na\(^{+}\) channel (see section 1.7.4). Carbamazepine at a molecular level has its effects on the Na\(^{+}\) channel in the same range as the therapeutic range seen in humans (Willow et al., 1985). The data presented by Shields and Koltzenburg (2010) indicated that, at carbamazepine levels within the therapeutic range in humans, the effect on the NEP should be detectable.

It is known that carbamazepine has an active metabolite carbamazepine-10,11-epoxide which has been shown to be biologically active (Tomson and Bertilsson, 1984; Bertilsson and Tomson, 1986). In chronic dosing, the level of the metabolite carbamazepine-epoxide is 10-50% of its parent compound carbamazepine. In order to investigate whether the effect on the NEP we predict seeing humans is due to carbamazepine, or its metabolite carbamazepine-epoxide, serum concentrations of both were determined in human participants. In order to assess whether carbamazepine-epoxide concentrations measured in humans may be detected in rodent nerve at the relevant level, the rat saphenous skin-nerve model is used to investigate the effect on the NEP of increasing doses of carbamazepine-epoxide.
Figure 2.1: Pilot data of carbamazepine on the nerve excitability profile in the rat skin nerve preparation. a) Effects of carbamazepine on the charge duration plot. b) Effect of carbamazepine on threshold electrotonus. Axes are labelled, including units, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000). Baseline recordings without drug are compared with differing concentration of carbamazepine. Baseline (●), carbamazepine 3µmol/L (▲), 10 µmol/L(△), 30 µmol/L(■) and 100 µmol/L(□). From Shields and Koltzenburg (2010).
2.2 Hypothesis 1 and 2

**Hypothesis 1** The effects of carbamazepine may be detected by the NEP of the peripheral nerve of healthy volunteers. In particular it is hypothesised that the NEP parameters of; S2 accommodation and rheobase will be seen to increase but the SDTC to decrease, after carbamazepine.

**Hypothesis 2** The effects of carbamazepine-epoxide can be detected by the NEP in rat sensory nerve. In particular it is hypothesised that high concentrations the effects of carbamazepine-epoxide will mimic those of carbamazepine, whereas at low concentrations no effect will be seen.

2.3 Rationale for utilising repetitive stimulation in combination with threshold tracking.

2.3.1 Origins of the concept of “Use-Dependence”

Hille (1977a) built on existing work of Strichartz (1973) and Courtney (1975) in describing "frequency- or use-dependent inhibition" in his seminal paper which proposed the modulated receptor hypothesis. In the first of two linked papers, Hille (1977b) used a myelinated nerve of the frog to examine the effects of high and low pH (pH 6 - 8.3) on the ability of amine anaesthetics to block Na$^+$ currents. Hille provided evidence that the local anaesthetic receptor was located on the inside of the membrane (furthering the work of Narahashi et al. (1970) and Frazier et al. (1970)). Working with frog myelinated fibres, he found that the hydrophobic, neutral form of compounds, e.g. lidocaine at high pH, were able to access the receptor compartment much more readily than when in their hydrophilic, cationic form at low pH; this was demonstrated by the onset time of Na$^+$ current block.

Hille (1977a) then went on to examine the effects of membrane holding potential and pre-pulse potential on the blocking effects of the amine anaesthetics, showing that their effects were strongly voltage dependent. Having done this he investigated the frequency of stimulation and again...
showed strong frequency dependent blocking for the charged amines but not the neutral benzocaine.

These observations of voltage-dependence and frequency-dependence, combined with the Hodgkin and Huxley model, lead Hille to propose the modulated receptor hypothesis. The modulated receptor hypothesis supposes that drugs have differing preferences for the different states of Na\(^+\) channels and that they have on- and off-rates by which they can bind and unbind specific states, this is depicted graphically in Figure 2.2. From these properties of selective state affinity, and on- and off-rates, Hille argues that one can explain the observations he reports and the differences between drugs. A comment of Hille’s reflects the intricacies of drug action and is an early insight that the properties he describes, may bear relevance to therapeutic effect: “These complexities create a problem of even defining what should be meant by the potency of a local anesthetic drug in such measurements and suggest that reported parameters like "half-blocking concentrations" will vary from laboratory to laboratory. As already pointed out by Courtney (1974, 1975), frequency and use-dependent inhibitions limit the maximum rate of firing or the minimum interspike interval of a nerve or muscle cell and may play a role in specific anti-arrhythmic and analgesic actions.”

The concepts and evidence leading up to the modulate receptor hypothesis are reviewed by Wang and Strichartz (2012). Clinical use of drugs with preference for the fast inactivated state of Na\(^+\) channels are as cardiac class 1 antiarrhythmics, local anaesthetics, analgesics and anticonvulsants. Recently, a first in class compound to be brought into clinical use for the treatment of epilepsy is Lacosamide which preferentially affects slow inactivation of Na\(^+\) channels expressed in rat cortical neurons (Errington et al., 2008; Kellinghaus, 2009).

### 2.3.2 Modern study of “Use Dependence” and other characteristics of sodium channel blockers

Huang et al. (2006) have developed a high throughput optical membrane detection and electrical stimulation system sensitive for aspects of sodium channel blockade, including use dependence. Aside from providing a valuable new technique, the authors also identified sodium channel blocking effects of a large number drugs not traditionally considered
to have their mode of action as use-dependent sodium channel sodium channel blockers, such as a large number of antidepressants. Of 400 drugs surveyed, Huang et al. (2006) found 25% caused at least 60% inhibition of sodium currents at 10 \( \mu \text{M} \) concentration, a surprising finding, and one which the authors used to hypothesise that part of the therapeutic effect of these drugs may be through their sodium channel inhibiting properties.

Recognising that a large number of therapeutic drugs may be having action through their use dependent sodium channel blocking actions, prompted Lenkey et al. (2010) to use an automated patch clamp system to characterise in a comparable way a range of common therapeutic drugs. This approach addresses the concern of Hille that a simple measurement of report of half-blocking concentration may vary from laboratory to laboratory and only provide a partial insight into drug properties. The stimulation procedures they used and variables calculated to classify each drug are shown in Figure 2.3.

The approach also provides a starting point whereby biophysical parameters of drug action may be used to assess therapeutic potential in
a directly comparable way. The authors described three main categories of sodium channel blockers: 1) High potency, slow onset and offset kinetics, partial reversibility and use-dependence; 2) low potency, fast kinetics and almost full reversibility; and 3) high potency, very slow kinetics, apparent irreversibility and no use-dependence.

Lenkey et al. (2010) conclude “the correlations of inhibition properties both with chemical properties and therapeutic profiles would not have been evident through the sole determination of IC50; therefore, recording multiple properties of inhibition may allow improved prediction of therapeutic usefulness.”

The same group Karoly et al. (2010) went on to provide a mathematical model explaining the diversity of blocking characteristics described by Lenkey et al. (2010) with interesting results as will be described in chapter 8.

2.3.3 Applying use dependence to myelinated nerve

Much modern electrophysiology takes place on in-vitro systems with a patch clamp technique. With this method a wealth of knowledge has been gained, however the pathology and symptoms which sodium channel blockers aim to provide therapeutic benefit for take place in in-vivo systems with intact and function myelinated (or unmyelinated) nerve fibres.

There is a lack of techniques which are able to provide comparable information to cellular electrophysiology in a living system as has been outlined. While nerve excitability studies have provided a bridge for this gap in some respects, the phenomenon of use dependence is not well assessed by the standard nerve excitability protocols in their current form.

Noto et al. (2011) have recently used a single fibre stimulation technique to assess latency changes associated with repetitive stimulation of human motor nerve. The authors report increasing latency, and by inference threshold, with increasing numbers of stimuli and frequency. They explain their findings by activation of the electrogenic sodium potassium pump. Limitations of this technique are; its difficulty in maintaining the experimental setup, the effect of the muscle fibre velocity recovery function on the measured waveforms, as well as the contribution of the neuro-
muscular junction and muscle fibre to the conduction time. Indeed in a commentary on the article by Bergmans (2012), argues that the prime determinant of the latency changes measured is likely to be the muscle fibre velocity recovery function.

In this study I proposed to utilise threshold tracking techniques to study the frequency dependent threshold properties on myelinated nerve in the rodent skin-nerve system (myelinated nerve ex-vivo) and in human sensory and motor nerve and the effects of drugs upon these properties.

2.4 Hypothesis 3 and 4

**Hypothesis 3**  The repetitive stimulation of myelinated nerve will increase threshold of the nerve in a frequency dependent manner and may be measured by the technique of threshold tracking.

**Hypothesis 4**  The use dependent characteristics of three different sodium channel blockers; lidocaine, carbamazepine and tetrodotoxin may be quantified using the repetitive stimulation and threshold tracking in the rodent skin-nerve preparation.
Figure 2.3: A modern approach to automatedly quantifying sodium blocking properties of drugs with an automated patch clamp system from Lenkey et al. (2010).

Original legend: Calculation of parameters and examples for the different types of inhibition caused by sodium channel inhibitors. A)–D) Peak amplitudes of evoked currents (5 Hz trains of 5 depolarizations from -90 to -10 mV) are plotted against time. Black dots: Control. Grey dots: Drug perfusion. A) “Type 1” inhibition (high potency, slow kinetics, partial reversibility, use-dependence). Calculation of properties of inhibition is illustrated. Inhibition: $Inh = (A_1 - A_3)/A_1$; $IC_{50} = (1 - Inh) \times cc/Inh$, where “cc” is the concentration; Reversibility: $Rev = A_5/A_1$; Use-dependence: $UD = (A_3/A_4)/(A_1/A_2)$; $\tau_{on}$ and $\tau_{off}$ are determined by mono-exponential fitting of peak amplitudes of the first evoked current in each train. B) Use-dependent “Type 2” inhibition (low potency, fast kinetics, good reversibility, use-dependence). C) Non-use-dependent “Type 2” inhibition (low potency, fast kinetics, good reversibility, no use-dependence). D) “Type 3” inhibition (high potency, very slow kinetics, apparently irreversible, no use-dependence). E) Calculation of $K_r$ and $K_i$ values from steady-state availability curves. CBZ - carbamazepine, DMI - desipramine, FLR - flunarizine, TOL - tolperisone.
Chapter 3

Methods

This chapter explains the methods by which the stated hypotheses will be tested. It explains how the method developed by Joseph Bergmans and later Hugh Bostock has been implemented in the context of a clinical trial of healthy human subjects taking a dose of carbamazepine. This is allied with description of the method in which the same technique can be applied to a dissected rat saphenous nerve in an ex-vivo environment. The method of adapting the current nerve excitability testing software to allow for testing frequency dependence are then detailed.

3.1 Skin Nerve Preparation

The skin-nerve preparation was used for in vitro recording as first described by Reeh (1986) and then further by Kress et al. (1992) as utilised by Maurer et al. (2007). A picture of the preparation used in this investigation is shown in Figure 3.1 and described below.

Adult female Sprague-Dawley rats were killed by cervical dislocation. The hind limb and pelvic skin was shaved to remove excess fur. The saphenous nerve was exposed and dissected from the inguinal ligament proximal to the epigastric artery, where it was cut and tied, to its insertion in the skin of the hind limb. The skin of the lower hind limb along with the saphenous nerve was removed with its insertion into the skin, and thus innervation, remaining intact. The dissected preparation consisted of a section of whole purely sensory nerve (the saphenous nerve has no motor component) between 40 and 50mm in length with an attached area of innervated hindlimb skin measuring approximately 30mm by 40mm.
The preparation was mounted in a two chamber organ bath with the skin pinned down corium side up (hairy skin side down) to the silicone (sylgard) base of the first (main) chamber. The saphenous nerve was then guided into a secondary chamber and rested on a gold wire active recording electrode which was electrically isolated from the main bath by bathing in liquid paraffin. A gold reference electrode was located nearby the paraffin and associated nerve segment in the fluid of the organ bath. A ground electrode was placed at a further distance in the organ bath fluid. Amplification was via a purpose built pre-amplifier and amplifier with an overall gain of 1000, low pass filter of 1kHz and high pass filter of 1Hz. The output signal was fed into an oscilloscope and the stimulating and recording system described in section 3.3.

The preparation was bathed in synthetic interstitial fluid (SIF) (Bretag, 1969; Maurer et al., 2007) consisting of, in mmol, 123 NaCl, 3.5 KCl, 0.7 MgSO$_4$, 1.7 NaH$_2$PO$_4$, 2.0 CaCl$_2$, 9.5 sodium gluconate, 5.5 glucose, 7.5 sucrose and 10 HEPES, titrated to a pH of 7.40. The SIF was perfused with oxygen and maintained at a temperature of 32 °C via a countercurrent heat exchanger surrounding the flow of SIF into the organ bath.

A portion of nerve to be stimulated was isolated in the main chamber using a pair of stainless steel rings one inside the other (outer diameter 14mm and 10mm respectively, wall thickness 1mm, height 14mm). The rings were placed over the top of the nerve and abutting the corium providing a fluidic seal from the surrounding SIF. A small notch was cut from the bottom side of both rings to provide a channel for the nerve to exit and avoid pressure effects but maintain a fluidic seal. SIF was pipetted out of the middle chamber of the outer ring before placement of the inner ring to inspect for and ensure a fluidic seal. The narrow space between the rings was filled with liquid paraffin oil to provide the electrically isolated central compartment such that current flow from the cathodal stimulation electrode would travel as much as possible via the nerve and not be short circuited via the SIF and metal rings to the anode. The central chamber of the inner ring was then inspected, to ensure a fluidic seal, for leakage of oil.

The chamber created in the middle of the rings was perfused with SIF via a separate circulation system and a stimulating Ag/AgCl electrode, cathode, (EPO5, World Precision Instruments, Hertfordshire, UK, uninsulated tip diameter 2mm) was lowered to between 1-2mm over
Figure 3.1: The skin-nerve preparation Dissected rat hind limb skin with saphenous sensory nerve attached is secured to the base of the main chamber. The nerve (highlighted in green to aid visualisation) is lead under the two metallic tubes of the stimulation and drug chamber. Paraffin oil is instilled between the two metallic tubes to provide electrical insulation. Into the central chamber formed by the two tubes, the stimulating electrode is lowered into this chamber together with a fine plastic drug delivery tube and a suction tube which provide a constant circulation of synthetic interstitial fluid with or without drug. The nerve exits from underneath the metal tubes and is guided into the recording chamber which is separated by a plastic divider and has a layer of paraffin oil floating on the synthetic interstitial fluid. A gold wire recording electrode lies in the oil layer onto which the nerve is laid. The bathing fluid is circulated in the main chamber and the stimulating chamber by separate pumps to maintain a constant level.

The anode, an electrode of the same type, was placed 10-30mm outside of the rings in a location that minimised stimulus artefact recorded (determined by adjustment during control recordings). Alongside the stimulating electrode in the chamber in the middle of the rings were secured a fine plastic tube to deliver a drug solution along with a fine plastic tube for removal of the drug solution. Drug solution or control SIF was circulated in the chamber using a delivery pump and removal pump set to maintain a constant fluid level within the chamber and using a distant reservoir from which this fluid was picked up or returned by the pumps.
3.2 Human sensory and motor NEP recording

After informed consent and trial enrolment (described in section 3.4), participants were seated in a soft chair and their non-dominant forearm rested in a maintainable, relaxed and comfortable position on the soft arm rest of the chair. A thermostatic warming blanket was applied to (wrapped around) the forearm and maintained the surface temperature of the skin at 35 °C. The warming blanket also served to stabilise the forearm further and reduce the chance of minor movement, if necessary a length of adhesive medical tape was used to provide a further anchor and prevent movement.

Stimulation for both motor and sensory recording was applied via non-polarisable surface electrodes (Red Dot, 3M Health Care, Loughborough, England), the active electrode placed over the median nerve at the wrist with the reference electrode approximately 10cm proximal over the the forearm but off the course of the median nerve to the lateral aspect of the volar forearm. Stimulating current was applied via an isolated linear bipolar constant current stimulator (maximum output +/-50mA, DS5, Digitimer, Welwyn Garden City, Herts, UK) and controlled by the QTRAC computer software.

For motor recording, the median nerve compound muscle action potential was recorded with electrodes over the abductor pollicis brevis muscle of the thenar eminence with a muscle belly tendon montage (see Figure 3.2), for sensory recording the sensory nerve action potential was recorded from digit 2 with electrodes separated 4cm centre to centre. A ground electrode was placed over the dorsum of the hand. Kendall 5400 surface electrodes (Covidien, Mansfield, Massachusetts, USA) were used for both muscles and sensory nerve recording. The electrical signal was amplified (gain 60 for motor recording and 30k for sensory recording) with a Nicolet Biomedical EA-4 amplifier (Natus Europe GmbH, Planegg, Germany), band pass filtered 10Hz-10kHz for motor recordings and 30Hz-3kHz for sensory recordings, and digitised (National Instruments BNC-2110 data acquisition device). An inline noise reduction device, Quest Scientific Hum Bug (Digitimer, Welwyn Garden City, Herts, UK), was used to reduce mains interference. The digitised signal was analysed by the computer with the recording, stimulation and analysis software, QTRAC copyright Institute of neurology, London (see...
section 3.3 for description).

![Figure 3.2: Electrode setup for recording the compound muscle action potential from the abductor pollicis brevis muscle. Surface recording electrodes are placed over the belly and tendon insertion of the muscle. Stimulating electrodes placed over the median nerve at the wrist and 10cm proximal. The proximal electrode is placed lateral to the course of the median nerve, in order to minimise stimulation at this site, due to it acting as a cathode during some protocols. Electrodes placed over the 2nd digit enable recording of the median nerve sensory nerve action potential when connected to the recording leads with stimulation again at the wrist (antidromic recording).](image)

### 3.3 Stimulation and Recording with QTRAC

The NEP was recorded using the TROND protocol as described by Kieran et al. (2000) and was used in both human and animal studies. A modification to this protocol was made to prolong the pulses conditioning pulses used in depolarising and hyperpolarising threshold electrotonus and to the recovery cycle as described in sections 3.3.4 and 3.3.6 respectively.

The stimulation and recording software QTRAC (copyright Institute of Neurology, London) and written by Professor Hugh Bostock was used to deliver stimuli via constant current stimulators and to display and analyse the signal from the respective amplifiers for human and skin-nerve
experiments as described in sections 3.1 and 3.2. QTRAC is a quanti-
tative threshold tracking and flexible stimulus response program de-
veloped by Professor Hugh Bostock at the Institute of Neurology, London.
Figures 3.3 to 3.8 illustrate this technique and its resulting data; the data
plotted represents the mean (solid lines) and standard deviation (broken
lines) of 29 normal controls from Kiernan et al. (2000). The NEP param-
eters presented in the results section as indices of nerve excitability are
derived from these curves and are described in the following sections.

The TROND protocol consists of a series of conditioning stimulus,
test stimulus combinations:

3.3.1 Stimulus-response relationship

The stimulating current is increased from zero to a level where a supra-
maximal nerve or muscle response obtained from the recording elec-
trodes. The stimulus is then reduced in a regular fractional amount of that
supra-maximal stimulating current in order to create a stimulus response
curve. The threshold current is then defined as the stimulus strength re-
quired to elicit a 40% maximal response (see Figure 3.3). The slope of
this plot is the NEP variable stimulus-response slope or SRSlope.

3.3.2 Threshold tracking

Once a control threshold is established from the stimulus-response re-
lationship, under varying conditioning stimuli, or variations in the condi-
tions of the nerve, the strength of stimulus required to elicit the desired
40% maximal response may change. A method is required to adjust the
stimulus to measure the new threshold i.e. there is a threshold change.

The method used is one of computer controlled threshold tracking.
The error between the desired response (i.e. predetermined 40% max-
imal response) and the measured response is calculated, and the stim-
ulus current is adjusted in proportion to this error by the computer until
it is once again sufficient to elicit the desired response, thus the term
“threshold tracking”. The magnitude of the correction needed (the track-
ing steps) is determined by the slope of the stimulus response curve at
the threshold point, SRSlope, but the magnitude of the tracking steps
can also be adjusted manually in the program in case of over or under-
Figure 3.3: Stimulus Response Plot. The threshold is defined as the current necessary to elicit a 40% maximal response. The proportion of maximal response is plotted against the stimulating current expressed as a proportion of threshold. The maximal slope of this curve is used to aid the threshold tracking software in predicting the step in stimulation current required to achieve the target response size. Data plotted represents the mean (solid lines) and standard deviation (broken lines) of 29 normal controls from Kiernan et al. (2000)
shoot by undesirable amounts. Figure 3.4 illustrates a test pulse eliciting a threshold response from the nerve. A conditioning pulse is then added, in this case a depolarising electronic pulse, the test pulse then produces a supra maximal response and must be adjusted in order to measure threshold under this new condition. This is done and a new stimulus strength or threshold is found which elicits the desired response. The difference between the control threshold and the conditioned threshold is referred to as threshold change and is expressed as a percentage of the control threshold.

![Threshold tracking diagram](image)

**Figure 3.4:** Threshold tracking. a) A control threshold (upper trace) and target response is established (lower trace), typically 40% of maximal response. b) A submaximal conditioning pulse is added to the test pulse changing the response away from the desired target response. c) The variation of the response size away from the target is observed by the threshold tracking system and an adjustment made in the “conditioned” threshold in order to elicit the target response size once again. The difference between the control and conditioned threshold is referred to as the threshold change. Threshold tracking is the process of adjusting the test stimulus to achieve the correct target response.

### 3.3.3 Strength-duration relationship

The strength-duration relationship is plotted by adjusting the duration of the rectangular stimulating current pulse (Figure 3.5a). At each duration the threshold or stimulus strength required to elicit the desired response (40% maximal) is obtained via threshold tracking. The threshold is measured at stimulus durations of 0.1, 0.2, 0.3, 0.4 and 0.5ms, and the threshold charge plotted against stimulus duration. The resulting plot shows a straight line in keeping with Wiess’ Law (refer to Theorem 1.1)
Weiss (1901). The intercept with the abscissa indicates the value of the SDTC and the slope indicates the rheobase as defined by Lapique (Theorem 5) Lapicque (1909). Figure 3.5 illustrates this plot and indicates the NEP parameters derived from it, SDTC and rheobase.

![Figure 3.5: Charge Duration Plot](image)

**Figure 3.5: Charge Duration Plot.**

a) To determine the charge duration (or strength-duration relationship) the duration of the test pulse is varied (time in ms on the x-axis) and in order to maintain the correct size target response (i.e. threshold tracking) the strength (current in mA on the y-axis) must also be varied. The area under the curve of the pulse is charge. The variation in height of the pulse represents the threshold tracking process adjusting the amplitude in order to achieve target response and is represented by the dotted lines.

b) The charge duration plot, charge (mA.ms) is plotted against test pulse duration (stimulus width in ms). The labels indicate the derived nerve excitability measures which are made on these curves. Data plotted represents the mean (solid lines) and standard deviation (broken lines) of 29 normal controls from Kiernan et al. (2000). Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).

**3.3.4 Threshold Electrotonus**

Threshold electrotonus is measured by conditioning the nerve with sub-threshold depolarising and hyperpolarising stimuli. Sub-threshold conditioning stimuli strengths are expressed as a proportion of the threshold current of the unconditioned nerve; 40% depolarising threshold electrotonus (TEd40), 20% depolarising threshold electrotonus (TEd20); 40%
hyperpolarising threshold electrotonus (TEh40) and; 20% hyperpolarising threshold electrotonus (TEh20). The duration of TEd40 and TEh40 was adjusted to 200ms, and the duration of TEd20 and TEh20 was 100ms. This was done to better allow insight at further time points of the effects of sodium channel blockers on the accommodative properties of the nerve. At the end of the conditioning pulse a further 100ms of threshold is measured in each condition to demonstrate the after effects of a polarising pulse. This demonstrates an undershoot and overshoot of the baseline, for depolarising and hyperpolarising conditioning respectively. Only 40% polarising threshold electrotonus conditions are shown in this thesis, as the 20% condition added no further information and plotting only 40% improves clarity of the plots.

Figure 3.6 shows how TE is plotted with delay in ms after the start of the conditioning pulse on the abscissa and the percentage reduction in threshold stimulating current required compared to unconditioned nerve on the ordinate. The abbreviations TEh refer to the hyperpolarising condition (or cath electrotonus) and TEd to the depolarising condition (or an electrotonus) with a number in percent added to illustrate the strength of the conditioning stimulus as a percentage on control threshold e.g. TEd40.

To clarify again, the threshold current is that required to elicit a 40% maximal nerve or muscle response and the conditioning pulses in electrotonus set to 40% of that value. In the case of this thesis, the 20% condition is omitted and thus this extra indication of percentage will be omitted and all conditioning pulses set to 40% of threshold current. Thus TEd refers to TEd40. A further indicator of time is added to indicate how long after the start of the conditioning pulse the excitability measurement is taken i.e. TEd(X ms) where X is the interval from the start of the conditioning pulse until measurement of threshold.

Illustrated in Figure 3.6, are the NEP parameters derived from threshold electrotonus namely; the peak threshold reduction in depolarising TE, TEd(peak); the threshold reduction measured at varying time points along the conditioning pulse e.g. TEh(90-100ms) and TEd(90-100ms); and the derived measure of S2 accommodation which refers to the degree of accommodation shown by the threshold reduction over the course of a depolarising pulse and measurement of which is indicated. The parameter accommodation half-time refers to the rate at which the accom-
3.3.5 Current-Threshold Relationship

The current-threshold relationship or IV curve, measures the threshold current at 200ms into a sub-threshold conditioning pulse, which is varied in fine increments from strongly hyperpolarising to depolarising. The threshold reduction is plotted on the abscissa and is the whole nerve analogy, of the membrane voltage. A positive threshold reduction as plotted by convention, indicating a depolarised membrane and a negative threshold reduction indicating a hyperpolarised membrane. The variation in conditioning current as a percentage of threshold is then plotted against this threshold reduction and the resulting curve gives insight into membrane conductance and rectifying properties of the nerve. Figure 3.7a) illustrates this plot; Figure 3.7b) indicates how the conditioning current is varied but the test pulse remains at a set delay at which it measures threshold reduction. Derived NEP parameters from this plot are; the slope of the IV curve which is an analogy for conductance of the membrane, the resting IV slope and hyperpolarising IV slope. Resting IV slope indicates conductance of the nodal membrane at rest.

3.3.6 Recovery cycle

The recovery cycle uses a supra-maximal conditioning stimulus followed by a test stimulus to measure threshold at varying time intervals between 2 and 200ms afterwards in humans and 0.44 and 200ms in rat nerve (a modification of TROND in rodent nerve due to the reduced refractory time). In order to acquire an accurate representation of the response elicited by the test stimulus, the response from a supra-maximal stimulus alone is digitally subtracted from the response from the combined conditioning and test stimuli. This process is necessary at shorter intervals due to contamination of the conditioned test response by the conditioning pulse response.

Figure 3.8a shows how the recovery cycle is then plotted with inter-stimulus interval on the abscissa and the threshold change as a percentage of unconditioned threshold as the ordinate. Figure 3.8b illustrates how the test pulse varies in delay after the conditioning supra maximal
Figure 3.6: Threshold Electrotonus. a) Plots threshold reduction as a percentage of control threshold (a reduction in threshold may be thought of as an increase in excitability) against the delay of the test pulse as measured in ms from the start of sub-threshold depolarising conditioning pulse as shown in b). b) shows how the test pulse is moved along the conditioning pulse and adjusted to find the correct conditioned threshold at that delay (dotted line). Plot c) and d) show the equivalent for a hyperpolarising conditioning pulse. The labels indicate the derived nerve excitability measures which are made on these curves. Data plotted represents the mean (solid lines) and standard deviation (broken lines) of 29 normal controls from Kiernan et al. (2000). Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).
Figure 3.7: Current-Threshold or IV Curve. a) plots the current of a prolonged submaximal conditioning pulse as a percentage of control threshold, against the reduction in measured threshold when the conditioning current is applied. b) illustrates the pulse configuration plotting current against time with the dotted line and arrow indicating the variation in conditioning current strength. The threshold gives an insight into the membrane voltage and an analogy can be made between this plot and the IV plot familiar from patch clamp work. Data plotted represents the mean (solid lines) and standard deviation (broken lines) of 29 normal controls from Kiernan et al. (2000). Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).
pulse to produce this plot. Derived NEP parameters from this plot are the relative refractory period (RRP), refractoriness indicated by the amount of threshold increase seen in time intervals below the RRP, the early superexcitability and the late subexcitability. Superexcitability and subexcitability are measured at the maximum points of change from baseline, but can also be measured at particular time intervals after the conditioning pulse in which case this time interval is indicated.

(a) Recovery Cycle

(b) Pulse configuration

Figure 3.8: Recovery Cycle. A single supra-maximal brief conditioning current is applied, threshold is then measured at varying time points after this conditioning pulse. a) plots threshold change as a percentage proportion of control threshold against the interstimulus interval between the conditioning and test pulse in ms. It should be noted that, by convention, the ordinate in this plot is at odds with other graphical depictions in the nerve excitability protocol, in that an increase in excitability is represented by a decrease in the ordinate, this is the opposite to the other plots seen. b) plots current against time to indicate the pulse configuration of the conditioning pulse (first) and test pulse (second), the dotted line and arrow indicates an increase in the interstimulus interval and resultant change in tracked threshold. Data plotted represents the mean (solid lines) and standard deviation (broken lines) of 29 normal controls from Kiernan et al. (2000). Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).
3.4 Recruitment and trial protocol for carbamazepine in humans

The study took place in the Department of Clinical Neurophysiology of the National Hospital for Neurology and Neurosurgery, UCLH NHS Foundation Trust, Queen Square, London, WC1N 3BG. Ethical approval was given by the Northwest London Research Ethics Committee 2 and the study conformed with the Declaration of Helsinki.

Volunteers were recruited and screened for inclusion and exclusion criteria of the study, screening criteria are shown in Table 3.1 to ensure health, minimise risk from adverse drug reactions and ensure suitable for recording of the NEP. 12 healthy volunteers were recruited, 3 women and 9 men (age range 20-42 years, mean 27 years) and all participants gave written informed consent.

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-65 years of age</td>
<td>sensitivity/idiosyncrasy to carbamazepine</td>
</tr>
<tr>
<td>history of good mental and physical health</td>
<td>Chinese, Malay, Indonesian or Thai origin</td>
</tr>
<tr>
<td>body mass index (BMI) &lt;30 &gt;18kg/m²</td>
<td>donation of blood in last 30 days</td>
</tr>
<tr>
<td>heart rate &lt;100 &gt;50 bpm</td>
<td>relevant medical disorder</td>
</tr>
<tr>
<td>normal blood pressure</td>
<td>history of A-V conduction abnormalities</td>
</tr>
<tr>
<td>normal electrocardiogram (ECG)</td>
<td>history of bone marrow depression</td>
</tr>
<tr>
<td>normal full blood count</td>
<td>history of acute porphyria</td>
</tr>
<tr>
<td>normal liver function test</td>
<td>history of long-QT syndrome</td>
</tr>
<tr>
<td>normal baseline nerve excitability profile</td>
<td>QTC interval &gt;450ms ♂ or &gt;470ms ♀</td>
</tr>
<tr>
<td>written informed consent</td>
<td>history of drug abuse</td>
</tr>
<tr>
<td></td>
<td>positive urinary drug screen</td>
</tr>
<tr>
<td></td>
<td>positive pregnancy test or breastfeeding</td>
</tr>
<tr>
<td></td>
<td>enzyme inhibiting medication in the last 30 days</td>
</tr>
<tr>
<td></td>
<td>use of any other systemic drug in past 7 days</td>
</tr>
<tr>
<td></td>
<td>participation in drug trial in last 30 days</td>
</tr>
<tr>
<td></td>
<td>more than mild carpal tunnel syndrome</td>
</tr>
<tr>
<td></td>
<td>hyperhidrosis</td>
</tr>
<tr>
<td></td>
<td>cutaneous lesions at stimulation or recording site</td>
</tr>
</tbody>
</table>

Table 3.1: A table showing the screening (inclusion and exclusion criteria) for the study of carbamazepine on the nerve excitability profile in healthy human volunteers. Chinese, Malay, Indonesian or Thai origin is an exclusion criteria due to the prevalence of the HLA-B*1502 allele which is linked to hypersensitivity reactions to carbamazepine.

Participants attended three study visits, and were compensated financially for their time and expenses. Study visit one consisted of giving informed consent, and undergoing a medical consultation and tests as outlined in the screening criteria (Table 3.1).

Study visit two consisted of a whole day and took place after suc-
cessful screening of the participant. Baseline (pre-drug) motor and sensory NEPs were recorded and a vein cannulated for serum drug level sampling. Tegretol liquid, a carbamazepine oral suspension, 10mg/kg was administered (supplied by the pharmacy at the National Hospital for Neurology and Neurosurgery). The motor and sensory NEP was then recorded at three time points throughout the day at 2, 4 and 8 hours after drug administration. Blood samples were taken from the venous cannula at 0.5, 1, 2, 4 and 8 hours post drug administration in order to measure serum carbamazepine and carbamazepine-epoxide concentration. Adverse events were enquired for and recorded if present.

Study visit three took place the following day at 24 hours post drug administration, a final motor and sensory NEP was recorded along with a final blood sample for drug concentration measurement. Adverse events still present were recorded and the history of adverse events clarified form the previous day if any occurred.

A follow up telephone call took place between 5 and 12 days after study medication for recording of any relevant medical history in the intervening period and any further adverse events.

Link code anonymised blood samples were sent to the Prof Patsalos Therapeutic Drug Monitoring Unit at the Chalfont Centre for Epilepsy, Chesham Lane, Chalfont St Peter, Bucks, for measurement of serum carbamazepine and carbamazepine-epoxide concentration.

Link code anonymised study data was stored in a paper file in the principal investigator’s office with a separate paper file providing the link codes back to identifiable information (should future contact be required) stored securely and separately by the principal investigator. Anonymised electronic data was analysed by computer.

3.5 Drugs used in human and rodent nerve

3.5.1 Carbamazepine oral suspension 10mg/kg was calculated to achieve therapeutic range in human volunteers

Carbamazepine oral suspension 100mg/5ml (Tegretol Liquid, Novartis) was obtained from the pharmacy at the National Hospital for Neurol-
ogy and Neurosurgery, Queen Square, London. According to the BNF 61 Joint Formulary Committee (2011) the plasma concentration of carbamazepine for the optimal therapeutic response in adult patients with epilepsy is 4-12 mg/litre (approximately 20-50µmol/L) and the recommended maximal daily dose is 1,600 mg.

The dose of 10mg/kg was calculated using a volume of distribution of 1.2L/kg and oral bioavailability of 90% in the middle of the summary product characteristics parameter range for these values. This means for a 70kg person, the dose of carbamazepine administered would be 700mg and the plasma level expected to be achieved would be (independent of weight of the subject) approximately 30µmol/L, but maybe as high as 50µmol/L if the extremes of bioavailability and volume of distribution are assumed, i.e. at the top of the therapeutic range.

The dose of 10mg/kg therefore was predicted to achieve levels in our trial participants towards the upper end of the therapeutic range in which there is a clinical effect of the drug which we hope to see neurophysiologically.

3.5.2 Carbamazepine, carbamazepine-epoxide and tetrodotoxin were diluted in SIF for administration to rodent nerve

Carbamazepine 10,11-epoxide >98% pure, carbamazepine >98% pure and Lidocaine >98% pure (Sigma-Aldrich Company Ltd., Dorset, England) and tetrodotoxin >98% pure (Alamone Labs, Jerusalem, Israel) were obtained in powder form. Carbamazepine and carbamazepine-epoxide were dissolved in dimethyl sulfoxide (DMSO) to make 100mmol/L stock which was refrigerated for storage. This stock was then diluted with SIF to make working solutions that contained no more than 0.1% DMSO. Tetrodotoxin was dissolved in DMSO then diluted with modified SIF to make working solutions that contained no more than 0.1% DMSO.

3.6 Data Analysis

Data acquired by the program QTRAC-S the stimulating, recording and threshold-tracking part of the program was then analysed offline by QTRAC-
P the data analysis portion of the software package. QTRAC-P enables plot generation of the NEP as presented here. QTRAC-P also allows statistical analysis of the NEP variables and was used for preparation of the results. Statistical data was analysed in QTRAC-P and values confirmed in the statistical program R (Team, 2012). Variables were tested for normality using Lilliefors test. Selected variables failed that normality test in which case the were transformed via a logarithm function and passed the normality test as log transformed data and further statistical analysis was performed on the log transformed data. To determine significant differences over the different time points before and after drug administration repeated measures one-way analysis of variance (repeated measures ANOVA) was used for each of the dependent variables. Posthoc tests were then performed as pairwise t-tests between time points with bonferroni correction where stated. Pharmacokinetic data was analysed in and single compartment modelling pharmacokinetic parameters computed from the data using the PKFit package (Lee and Lee, 2009).

3.7 Adaptation of the protocol to assess frequency-dependence

The stimulation and recording software QTRAC (copyright Institute of Neurology, London) was again used as described in section 3.3.

For these experiments a customised script was written for the program and the following protocol employed. A just supra-maximal test stimulus of width 0.2ms was found by manual stimulus adjustment. A stimulus response curve was measured. The control stimulus strength (or threshold) to elicit a 40% maximal response was found from the stimulus response curve. A 3 minute baseline period was measured to ensure stability of control threshold at stimulation rate of 2Hz, during this period the threshold was tracked by computer (stimulus adjusted to ensure consistent response of 40% maximal) as it was during the rest of the experiment.

Having acquired a baseline and control threshold, 500 stimuli were delivered at a set frequency, increasing between trials from 5-20Hz.

In human trials frequency categories of 5, 10 and 20Hz stimulation were used. In ex-vivo rat skin nerve experiments, frequency categories
of 5, 10, 15 and 20Hz stimulation were used, as it became clear from human experiments that there was a large change between 10Hz and 20Hz. The extra stimulation frequency of 15Hz was recorded in order to fill this gap.
Chapter 4

Results - Nerve Excitability Measures to Detect the Effects of Carbamazepine in Human Subjects

The aim of this chapter is to describe the results of the clinical trial of healthy human volunteers receiving a sufficient dose of oral carbamazepine to raise serum levels to the upper end of the therapeutic range. The pharmacokinetics of carbamazepine in our study group are described followed by the measured nerve excitability profile over the course of 24 hours post dose. The results for sensory and motor nerve are displayed separately, both showing very significant measurable changes which are notably different between sensory and motor nerve.

4.1 Demographics of human trial participants

22 volunteers were screened for inclusion into the trial using the criteria set out in table 3.1 (refer to Chapter 3.4 for details of methods). One participant voluntarily withdrew after the screening visit, nine volunteers failed screening criteria for reasons set out in table 4.1.

12 volunteers completed the study, 9 males and 3 females. Demographic data on participants are set out in table 4.2.
Table 4.1: A table showing the reasons trial volunteers did not progress to inclusion in the trial.

* The HLA-B*1502 allele is more common in certain ethnic groups and has been shown to be strongly associated with the risk of developing Stevens-Johnson syndrome when treated with carbamazepine.

Table 4.2: Demographic data of trial participants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>27 years (20-42)</td>
</tr>
<tr>
<td>Weight</td>
<td>72 kilograms (59-100)</td>
</tr>
<tr>
<td>Height</td>
<td>1.76 metres (1.56-1.86)</td>
</tr>
<tr>
<td>BMI</td>
<td>23.1 kg/m² (18.4-29.0)</td>
</tr>
</tbody>
</table>

4.2 Pharmacokinetics of carbamazepine in healthy volunteers

4.2.1 Carbamazepine serum levels achieve therapeutic range after a single oral dose of 10mg/kg.

The range for optimal clinical response (therapeutic range) for carbamazepine is a total serum concentration of 20-50 µmol/L (JointFormularyCommittee, 2011) which equates to a serum free concentration (non-protein bound) of 5-12.5 µmol/L assuming 75% protein binding. This study aimed to achieve total serum concentrations at the higher end of the therapeutic range after a single dose; the carbamazepine dose was calculated to achieve this concentration. Carbamazepine and carbamazepine-epoxide serum levels were measured at varying time points up to 24 hours post dose of 10mg/kg carbamazepine. The mean value of maximum measured concentrations of carbamazepine and carbamazepine-epoxide for each individual was 46 µmol/L (range of 39-54 µmol/L) and
2.1 \mu\text{mol/L} \text{ (range of 1-3.5 \mu\text{mol/L}) respectively. Mean serum concentration and 95% confidence intervals are plotted in Figure 4.1 grouped by time after dose.}

(a) Serum carbamazepine level  
(b) Serum carbamazepine-epoxide level

Figure 4.1: Mean total serum levels of a) carbamazepine (CBZ) and b) carbamazepine-epoxide (CBZe) in 12 healthy volunteers after 10mg/kg oral carbamazepine solution plotted against time after dose. Error bars indicate 95% confidence intervals. The curve represents a line of best fit for the grouped data. Therapeutic range of carbamazepine 20-50 \mu\text{mol/L}.

4.2.2 Pharmacokinetic modelling shows carbamazepine has a mean serum half life of 29.4 hours and a time for maximal concentration of 3.0 hours in drug naive individuals. These findings are comparable with published data.

Single compartment pharmacokinetic modelling of the data using the statistical program R and the package PKFit was used to determine best fit values for the carbamazepine pharmacokinetic values for each individual. Mean serum half life in the 12 participants was 29.4 h, mean time to maximal concentration (Tmax) 2.96 h, mean maximal modelled concentration (Cmax) 44.1 \mu\text{mol/L}, elimination rate (Ke) 0.0255 h\textsuperscript{-1}, ap-
parent volume of distribution 0.901 \, L/kg. These values are summarised in Table 4.3. These values are entirely in line with previously published data (Patsalos and Bourgeois, 2010; Gérardin et al., 1976; Tomson et al., 1983; Bertilsson and Tomson, 1986).

<table>
<thead>
<tr>
<th>Cmax (, \mu mol/L)</th>
<th>T1/2 (Hours)</th>
<th>Tmax (Hours)</th>
<th>Ke (h^{-1})</th>
<th>Vd (L/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean 44.1</td>
<td>29.4</td>
<td>2.96</td>
<td>0.0255</td>
<td>0.901</td>
</tr>
<tr>
<td>Min 37.0</td>
<td>16.8</td>
<td>1.60</td>
<td>0.0114</td>
<td>0.731</td>
</tr>
<tr>
<td>Max 50.0</td>
<td>60.8</td>
<td>7.10</td>
<td>0.0412</td>
<td>1.105</td>
</tr>
<tr>
<td>SD 4.6</td>
<td>10.6</td>
<td>1.52</td>
<td>0.0067</td>
<td>0.118</td>
</tr>
</tbody>
</table>

Table 4.3: Table showing a summary of single compartment modelled pharmacokinetic parameters in 12 healthy volunteers after 10mg/kg of oral carbamazepine solution. Cmax - Maximal concentration, T1/2 - Elimination half life, Tmax - Time to reach Cmax, Ke - Elimination rate, SD - Standard deviation.

4.3 Adverse Events in trial participants

Adverse events in trial participants were recorded according to the Common Terminology Criteria for Adverse Events (CTCAE) v4.03 (National Cancer Institute, 2009). The adverse events experienced by trial participants after 10mg/kg of oral carbamazepine are recorded in table 4.4. No unexpected adverse events, serious adverse events or suspected unexpected serious adverse events occurred. The CTCAE records different grades of adverse events; events are graded as mild (Grade 1), moderate (Grade 2), severe (Grade 3), life-threatening (Grade 4) or Death (Grade 5). Grade 1 and 2 events only were recorded in this study requiring no or minimal intervention.
<table>
<thead>
<tr>
<th>Adverse Event</th>
<th>CTCAE Grade (1-5)</th>
<th>Number Affected</th>
<th>Mean Duration (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somnolence</td>
<td>1</td>
<td>8/12</td>
<td>28 hours (0.75-120 hours)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2/12</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>10/12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ataxia</td>
<td>1</td>
<td>8/12</td>
<td>28 hours (5.75-120 hours)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1/12</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>9/12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysarthria</td>
<td>1</td>
<td>5/12</td>
<td>8 hours (2-24 hours)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dizziness</td>
<td>1</td>
<td>4/12</td>
<td>54 hours (22-120 hours)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>1</td>
<td>3/12</td>
<td>8 hours (1-24 hours)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1/12</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4/12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vomiting</td>
<td>1</td>
<td>2/12</td>
<td>30 minutes (20-40 minutes)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry Mouth</td>
<td>1</td>
<td>1/12</td>
<td>24 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pruritus</td>
<td>1</td>
<td>1/12</td>
<td>20 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Altered pitch perception</td>
<td>1</td>
<td>1/12</td>
<td>48 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.4:** A table showing adverse events occurring in trial participants classified according to CTCAE grade (NationalCancerInstitute, 2009). Somnolence was experienced by most volunteers being the most common adverse event. The CTCAE grade may range from 1 to 5, mild (Grade 1), moderate (Grade 2), severe (Grade 3), or life-threatening (Grade 4), Death (Grade 5). Tailoring of the grade to the specific symptom is given in the grading system.
4.4 The effects of carbamazepine on human nerve in vivo

The NEP was recorded prior to and then 2, 4, 8 and 24 hours after carbamazepine administration in 12 healthy participants.

4.4.1 Carbamazepine effect maybe detected in the Nerve Excitability Profile of human sensory and motor nerve

The results of the NEP were plotted graphically as outlined in the methods (Section 3.3). The changes between the baseline recording and at 4h after carbamazepine administration are shown graphically in Figures 4.2 and 4.3 for sensory and Figures 4.4 and 4.5 for motor nerve. The baseline and 4h time point are displayed to best demonstrate graphically the changes seen, other time points are not displayed in the figure for clarity but change over time in their variables is shown in figures 4.6 and 4.7. The plots show changes in the strength duration, depolarising threshold electrotonus, hyperpolarising threshold electrotonus, recovery cycle and I/V curve after carbamazepine (broken lines in the plots represent standard error of the mean). Interestingly the changes seen in the sensory NEP differ from those seen in the motor NEP most notably in the depolarising threshold electrotonus as will be discussed.

Repeated measures ANOVA was performed on the sensory and motor NEP variables (Tables 4.5 and 4.6 respectively). The most significant effects for both motor and sensory nerve were seen in the depolarising threshold electrotonus, with effects also seen on the I/V curve, recovery cycle and, for sensory nerve only, the strength duration curve.

4.4.2 Specific NEP parameters vary with time after carbamazepine and this variation is distinct between motor and sensory nerve

Figures 4.6 and 4.7 show how the NEP elements vary over time after dose of carbamazepine. The varying parameters were shown by repeated measures ANOVA described above, post-hoc comparison of
Figure 4.2: Effect of carbamazepine on human sensory nerve excitability. Sensory nerve excitability profile plots of a) charge duration, b) 40% depolarising threshold electrotonus (TE) c) 40% hyperpolarising TE and d) recovery cycle. Mean values in 12 healthy volunteers are plotted, before (blue line) and 4 hours after (green line) 10mg/kg oral carbamazepine. Dashed error lines indicate standard error of the mean. Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).
Figure 4.3: Effect of carbamazepine on human sensory nerve excitability. Sensory nerve excitability profile plots of a) stimulus response b) IV or current threshold curve c) expanded view of the depolarising segment of the IV curve and d) slope of the IV curve. Mean values in 12 healthy volunteers are plotted, before (blue line) and 4 hours after (green line) 10mg/kg oral carbamazepine. Dashed error lines indicate standard error of the mean. Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).
Figure 4.4: Effect of carbamazepine on human motor nerve excitability. Motor nerve excitability profile plots of a) charge duration, b) 40% depolarising threshold electrotonus (TE) c) 40% hyperpolarising TE and d) recovery cycle. Mean values in 12 healthy volunteers are plotted, before (blue line) and 4 hours after (red line) 10mg/kg oral carbamazepine. Dashed error lines indicate standard error of the mean. Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).
Figure 4.5: Effect of carbamazepine on human motor nerve excitability. Motor nerve excitability profile plots of a) stimulus response b) IV or current threshold curve c) expanded view of the depolarising segment of the IV curve and d) slope of the IV curve. Mean values in 12 healthy volunteers are plotted, before (blue line) and 4 hours after (red line) 10mg/kg oral carbamazepine. Dashed error lines indicate standard error of the mean. Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>F Value(degrees freedom)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control threshold</td>
<td>3.5(4, 44)</td>
<td>1.5×10⁻²</td>
</tr>
<tr>
<td><strong>Depolarising electrotonus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEd(190-200ms)</td>
<td>32.5 (4,44)</td>
<td>1.3×10⁻¹²</td>
</tr>
<tr>
<td>S2 accommodation</td>
<td>27.8 (4,44)</td>
<td>1.5×10⁻¹¹</td>
</tr>
<tr>
<td>Accommodation half-time</td>
<td>25.2 (4,44)</td>
<td>6.6×10⁻¹¹</td>
</tr>
<tr>
<td>TEd(90-100ms)</td>
<td>23.0 (4,44)</td>
<td>2.7×10⁻¹⁰</td>
</tr>
<tr>
<td>TEd(40-60ms)</td>
<td>6.4 (4,44)</td>
<td>3.6×10⁻⁴</td>
</tr>
<tr>
<td><strong>Hyperpolarising electrotonus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEh(10-20ms)</td>
<td>13.2 (4,44)</td>
<td>3.7×10⁻⁷</td>
</tr>
<tr>
<td>TEh(190-200ms)</td>
<td>11.2 (4,44)</td>
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<tr>
<td>TEh(90-100ms)</td>
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<tr>
<td>TEh(20-40ms)</td>
<td>6.4 (4,44)</td>
<td>3.8×10⁻⁴</td>
</tr>
<tr>
<td><strong>Charge duration</strong></td>
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<tr>
<td>Strength-duration\time constant</td>
<td>5.9 (4,44)</td>
<td>7.2×10⁻⁴</td>
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<tr>
<td>Rheobase</td>
<td>3.5 (4,44)</td>
<td>1.4×10⁻²</td>
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<tr>
<td><strong>IV curve</strong></td>
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<td>Resting I/V slope</td>
<td>10.2 (4,44)</td>
<td>6.4×10⁻⁶</td>
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<td>Minimum I/V slope</td>
<td>8.9 (4,44)</td>
<td>2.4×10⁻⁵</td>
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<tr>
<td>Hyperpol. I/V slope</td>
<td>5.8 (4,44)</td>
<td>8.1×10⁻⁴</td>
</tr>
<tr>
<td><strong>Recovery cycle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superexcitability (%)</td>
<td>9.2 (4,44)</td>
<td>1.8×10⁻⁵</td>
</tr>
</tbody>
</table>

**Table 4.5:** ANOVA analysis of individual human sensory NEP with repeated measures over time. Only parameters showing significant variation with repeated measures are shown. Significant changes were seen in all testing protocols for sensory nerve.
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<tr>
<th>Parameter</th>
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<th>P value</th>
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</thead>
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</tr>
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<td>TEd(40-60ms)</td>
<td>59.7 (4,44)</td>
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</tr>
<tr>
<td>TEd(peak)</td>
<td>45.1 (4,44)</td>
<td>$5.05 \times 10^{-15}$</td>
</tr>
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<td>TEd(190-200ms)</td>
<td>35.6 (4,44)</td>
<td>$2.8 \times 10^{-13}$</td>
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<tr>
<td>TEd(90-100ms)</td>
<td>23.7 (4,44)</td>
<td>$1.6 \times 10^{-10}$</td>
</tr>
<tr>
<td>TEd(10-20ms)</td>
<td>22.5 (4,44)</td>
<td>$3.5 \times 10^{-10}$</td>
</tr>
<tr>
<td>S2 accommodation</td>
<td>7.4 (4,44)</td>
<td>$1.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>Accommodation half-time</td>
<td>17.7 (4,44)</td>
<td>$9.9 \times 10^{-9}$</td>
</tr>
<tr>
<td><strong>Hyperpolarising electrotonus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEd(190-200ms)</td>
<td>7.8 (4,44)</td>
<td>$7.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>TEd(90-100ms)</td>
<td>6.8 (4,44)</td>
<td>$2.3 \times 10^{-4}$</td>
</tr>
<tr>
<td><strong>Charge Duration</strong></td>
<td></td>
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</tr>
<tr>
<td>No significant changes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IV curve</strong></td>
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</tr>
<tr>
<td>Resting I/V slope</td>
<td>5.7 (4,44)</td>
<td>$8.6 \times 10^{-4}$</td>
</tr>
<tr>
<td><strong>Recovery cycle</strong></td>
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<td></td>
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<tr>
<td>Superexcitability</td>
<td>19.7 (4,44)</td>
<td>$2.3 \times 10^{-9}$</td>
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<tr>
<td>Relative refractory period</td>
<td>3.6 (4,44)</td>
<td>$1.1 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

**Table 4.6:** Repeated measures ANOVA analysis of individual human motor NEP with repeated measures over time. Only parameters showing significant variation with repeated measures are shown. No significant changes were seen in charge duration measures for motor nerve.
means and paired t-tests were carried out to determine which time points showed the greatest change. The elements which vary after carbamazepine are discussed individually, a summary of the changes seen on the NEP as a result of carbamazepine are shown in table 4.7.

Figure 4.6: Post-hoc paired t-test against time 0h of selected sensory nerve excitability parameters, mean and standard error values in 12 healthy volunteers before (0h) and up to 24 hours after 10mg/kg oral carbamazepine. Against time in hours are plotted a) control threshold and the derived excitability parameters b) SDTC c) rheobase d) TEd(peak) e) S2 accommodation and f) superexcitability. NS-Not significant * p < 0.05 ** p < 0.01 *** p < 0.001. Scales are set to match those in the corresponding motor variables figure 4.7. Derived excitability variables are abbreviated and axis labelled including units where applicable in accordance with accepted nerve excitability convention as set out by Kiernan et al. (2000).

**Threshold,** the stimulus required to produce 40% supra maximal response, rises in sensory nerve but does not change in motor nerve after carbamazepine Threshold increased in sensory nerve after carbamazepine from a mean of 4.17mA at baseline to a maximum of 5.59mA at 4 hours post dose (p<0.01), in motor nerve however there
Figure 4.7: Post-hoc paired t-test against time 0h of selected motor nerve excitability parameters mean and standard error in 12 healthy volunteers before (0h) and up to 24 hours after 10 mg/kg oral carbamazepine. Against time in hours are plotted a) control threshold and the derived excitability parameters b) strength duration time constant (SDTC) c) rheobase d) TEd(peak) e) S2 accommodation and f) superexcitability. NS-Not significant * p<0.05 ** p<0.01 *** p<0.001. Scales are set to match those in the corresponding motor variables figure 4.7. Derived excitability variables are abbreviated and axis labelled including units where applicable in accordance with accepted nerve excitability convention as set out by Kiernan et al. (2000).
was no change in threshold (compare Figures 4.6a and 4.7a). For sensory nerve 24 hours after carbamazepine there remained a significant increase in threshold compared with baseline.

The strength duration curve shows a reduction in SDTC and increase in rheobase in sensory nerve but no change in motor nerve after carbamazepine. The SDTC reduced in sensory nerve from a mean of 0.485ms at baseline to a minimum of 0.415ms at 24 hours after carbamazepine (p<0.01) whilst rheobase increased from a mean of 1.92mA at baseline to a maximum of 2.79mA at 4 hours after carbamazepine (p<0.01) with the change persisting until the last time point of 24 hours after carbamazepine. In motor nerve no significant changes were seen in either SDTC or rheobase (compare Figures 4.6b and 4.7b).

Depolarising threshold electrotonus, with 40% conditioning stimuli, showed similar reductions in excitability at the end of the submaximal 200ms conditioning pulse in both motor and sensory nerve. The peak threshold reduction, however, was reduced in motor but not sensory nerve after carbamazepine. The threshold reduction at between 190 and 200ms (TEd190-200ms) was reduced from baseline by a maximum mean of 6.6% at 8 hours after carbamazepine (p<0.001) in sensory nerve and by a maximum mean of 5.0% at 4 hours (p<0.001) in motor nerve (Figures 4.2b and 4.4b respectively). The peak threshold reduction (TEd peak) in sensory nerve was unchanged (repeated measures ANOVA F=1.92 degrees of freedom 4,44 p=0.122) whereas in motor nerve the TEdpeak was reduced from baseline by a maximum mean of 6.3% at 4 hours after carbamazepine (p<0.001) (see Figures 4.6d and 4.7d respectively). The S2 accommodation is calculated as the reduction in threshold reduction (or to put it in more understandable terms, the decline in excitability) which develops over the period of the sub-threshold depolarising conditioning pulse (set as standard at 100ms but also can be optionally measured at 200ms. 200ms was utilised in order to increase the probability of inactivated sodium channel states developing). The S2 accommodation therefore increased from a mean at baseline of 16.3% to a maximum mean of 22.15% at 8 hours after carbamazepine (p<0.001) in sensory nerve, however in motor nerve the S2 accommodation decreased from a mean at baseline of 23.6% to a minimum mean
of 21.3% at 2 hours (p<0.01) (see Figures 4.6e and 4.7e respectively).

The recovery cycle shows a reduction in superexcitability in both sensory and motor nerve, and a reduction in the RRP for motor nerve only after carbamazepine. Superexcitability was reduced from a baseline mean of 17.0% to a minimum mean of 13.9% at 2 hours after carbamazepine in sensory nerve (p<0.001) and from a baseline mean of 24.2% to a minimum mean of 19.0% at 4 hours in motor nerve (p<0.001) (see Figures 4.6f and 4.7f respectively). RRP was unchanged in sensory nerve (repeated measures ANOVA F=0.799 degrees of freedom 4,44 p=0.53) but showed a minor reduction in motor nerve from a baseline mean of 2.95ms to a minimum mean of 2.61ms at 24 hours (p<0.05) after carbamazepine.

The I/V curve shows an increase in the resting I/V slope in both sensory and motor nerve along with an increase in hyperpolarising I/V slope in sensory nerve after carbamazepine. Resting I/V slope shows an increase from a baseline mean of 0.57 to a maximum mean of 0.69 at 8 hours after carbamazepine (p<0.001) in sensory nerve and from a baseline mean of 0.62 to a maximum mean of 0.69 at 4 hours (p<0.01) in motor nerve. The hyperpolarising I/V slope increased in sensory nerve only from a baseline mean of 0.34 to maximum mean of 0.39 at 8 hours after carbamazepine (p<0.01). See Figures 4.3b 4.3c 4.3d for sensory and 4.5b 4.5c 4.5d for motor IV curves and their slopes.

4.4.3 Relationship between serum carbamazepine level and the NEP

It was hypothesised that the NEP would vary directly with the carbamazepine serum level. Figure 4.8 shows the relationship between two main parameters of interest and the carbamazepine serum level grouped by time after the dose. Although there is significant linear correlation between carbamazepine serum concentration and S2 accommodation (Pearson’s R=0.65, p<0.001) the relationship, seen in Figure 4.8, appears to have a non-linear relationship with a time dependent parameter. Where as the relationship showing a weak correlation between carbamazepine serum level and superexcitability (Pearson’s R=0.26, p<0.05)
<table>
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<tr>
<th>Parameter</th>
<th>Sensory Nerve</th>
<th>Motor Nerve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threshold current</td>
<td>↑</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Depolarising electrotonus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEd(peak)</td>
<td>NS</td>
<td>↓</td>
</tr>
<tr>
<td>TEd(190-200ms)</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>S2 accommodation</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td><strong>Hyperpolarising electrotonus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T Eh(190-200ms)</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td><strong>Charge Duration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDTC</td>
<td>↓</td>
<td>NS</td>
</tr>
<tr>
<td>Rheobase</td>
<td>↑</td>
<td>NS</td>
</tr>
<tr>
<td><strong>IV curve</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting I/V slope</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Hyperpolarising I/V slope</td>
<td>↓</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Recovery cycle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative refractory period</td>
<td>NS</td>
<td>↓</td>
</tr>
<tr>
<td>Superexcitability</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

*Table 4.7:* A summary of the main effects of carbamazepine on human sensory and motor nerve excitability parameters, including the differences between the two.

NS - not significant on repeated measures ANOVA, † - Increase on post-hoc t-test, ↓ - Decrease on post-hoc t-test.
level appears linear. Complex relationships with time after dose also appear in other parameters of interest as illustrated in Figures 4.6 and 4.7. One possible explanation for this is that the concentration experienced by the nerve is related to the serum concentration through a time dependent phenomenon such as separate compartments, this is discussed more fully in the section 7.1.

4.4.4 Selected scatter plots separate individuals before and after carbamazepine with 100% sensitivity and specificity

Figure 4.9 shows scatter plots grouped by time after dose of carbamazepine and demonstrates that the NEP may provide 100% sensitivity and 100% specificity in distinguishing subjects before and after carbamazepine. The parameters sensitive to carbamazepine are shown to be different between motor and sensory nerve.

Summary

This chapter has detailed how carbamazepine’s effects maybe detected in the NEP of human sensory and motor nerve.

For both sensory and motor NEP, scatter plots of selected variables are able to separate individuals before and after carbamazepine with 100% sensitivity and specificity.

The changes in the profiles are seen to vary between sensory and motor nerve, for example the SDTC is reduced after carbamazepine in sensory nerve but no change is seen in motor nerve. Differences are also seen in the absolute threshold, threshold electrotonus curves, recovery cycle and IV curve. These results support Hypothesis 1, that the effects of carbamazepine can be detected by the NEP in the peripheral nerve of healthy volunteers. The hypothesised parameter changes are seen to be accurate for sensory but not for motor nerve.

The relationship between carbamazepine serum level and excitability parameters is shown not to be a straightforward linear one, but rather also dependent on the time after the dose.
Figure 4.8: Relationship between carbamazepine (CBZ) serum levels and selected sensory nerve excitability parameters. a) Plots the variation in S2 accommodation and b) superexcitability against carbamazepine serum level measured from 12 healthy volunteers before (0h) and up to 24h post an oral dose of 10mg/kg of carbamazepine. Ellipses indicate standard error of the mean. Dashed line indicates an estimated variation over time from 0 to 24 hours after drug. Derived excitability variables are abbreviated and axis labelled including units where applicable, in accordance with accepted nerve excitability convention as set out by Kiernan et al. (2000).
Figure 4.9: Selected variable scatter plots before and after carbamazepine (CBZ). Left hand column shows sensory nerve excitability parameters and right hand column shows motor nerve excitability parameters. a) and b) plot hyperpolarising I/V slope against S2 accommodation. c) and d) plot hyperpolarising I/V slope against resting I/V slope. e) and f) plot hyperpolarising I/V slope against TEd(40-60ms). Complete separation between groups before and after carbamazepine is possible with combinations of variables. Points represent individuals. Ellipse indicates 95% confidence interval. Cut-offs, dashed lines give 100% sensitivity and specificity. Derived excitability variables are abbreviated and axis labelled including units where applicable, in accordance with accepted nerve excitability convention as set out by Kiernan et al. (2000).
Chapter 5

Results - Nerve Excitability
Measures to Detect the Effects of Carbamazepine and Carbamazepine-Epoxide in Rat Nerve ex-vivo

This chapter aims to describe the effects of carbamazepine and also its metabolite carbamazepine-epoxide, when applied to the rodent saphenous nerve in the skin nerve preparation as measured by the NEP. In order to make comparison with the human trial participants, concentrations used in these experiments are related to serum levels in humans, taking into account protein binding.

The (by now familiar) standard plots of the NEP are shown for carbamazepine and carbamazepine-epoxide at low and high concentrations. Plotting the results according to nerve excitability convention allows direct visual comparison between experimental data. Comparison may be made by the reader between the human trial results presented in Chapter 4 and the results presented here. It should be remembered that the rodent saphenous nerve is a sensory not motor nerve.

5.0.1 Comparative drug concentrations in experimental work related to serum levels in trial participants

The mean maximal serum level achieved in humans of carbamazepine-epoxide was 2.1 µmol/L with a range of 1-3.5 µmol/L, and for carbamazepine,
was 44.1 \( \mu \text{mol/L} \) with a range of 37-50 \( \mu \text{mol/L} \). Carbamazepine-epoxide is known to be approximately 50% bound in humans (Gidal et al., 1996; MacKichan and Zola, 1984) meaning the effective free concentration is likely below 2 \( \mu \text{mol/L} \). For carbamazepine the mean free concentration equates to approximately 4.4 \( \mu \text{mol/L} \). In order to investigate both carbamazepine and carbamazepine-epoxide, both compounds were tested separately. Carbamazepine was applied to rodent nerve at concentrations of 0 \( \mu \text{mol/L} \), 3 \( \mu \text{mol/L} \), 10 \( \mu \text{mol/L} \), 30 \( \mu \text{mol/L} \), and for carbamazepine-epoxide, concentrations applied were 0 \( \mu \text{mol/L} \), 0.3 \( \mu \text{mol/L} \), 1 \( \mu \text{mol/L} \), 3 \( \mu \text{mol/L} \), 10 \( \mu \text{mol/L} \), 30 \( \mu \text{mol/L} \). A reduction in peak response (maximal recorded compound action potential) is seen with higher doses of both carbamazepine and carbamazepine-epoxide as shown in figure 5.1.

![Figure 5.1:](image)

**Figure 5.1:** Peak response of rodent sensory nerve in the skin nerve preparation after differing doses of a) carbamazepine and b) carbamazepine-epoxide. Peak response in mV is plotted against applied drug concentration in \( \mu \text{mol/L} \). At concentrations of carbamazepine of 30 \( \mu \text{mol/L} \) and carbamazepine-epoxide above 3 \( \mu \text{mol/L} \) a significant reduction in the peak sensory nerve response in the preparation was seen. NS-Not significant * \( p < 0.05 \).

### 5.1 The effects of carbamazepine on rodent nerve ex-vivo

7 individual adult Sprague-Dawley rat saphenous nerves were prepared and increasing doses of carbamazepine applied to the portion of nerve in the stimulating chamber as described in section 3.1.
5.1.1 Carbamazepine effect maybe detected in the Nerve Excitability Profile of rodent sensory nerve ex-vivo.

Repeated measures ANOVA, as shown in table 5.1, shows significant effects of carbamazepine on the NEP. The effects of carbamazepine on the NEP are shown graphically in figures 5.2, 5.3 and 5.4. At a carbamazepine concentration of 30µmol/L the IV curve could not reliably be recorded due to development of a depolarising block.

The main changes as a result of carbamazepine were upon the depolarising threshold electrotonus, hyperpolarising threshold electrotonus and recovery cycle. Notably in this study no significant changes were seen in the charge duration plot or the IV curve (with the caveat that depolarising block rapidly developed at the highest dose).

<table>
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<tr>
<th>Parameter</th>
<th>F Value(degrees freedom)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak response</td>
<td>8.8(3, 18)</td>
<td>8.9 ×10⁻⁴</td>
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<tr>
<td><strong>Depolarising electrotonus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEd(peak)</td>
<td>5.1(3, 18)</td>
<td>1.0 ×10⁻²</td>
</tr>
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<td>TEd(10-20ms)</td>
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<tr>
<td>TEd(40-60ms)</td>
<td>14.2(3, 18)</td>
<td>6.4 ×10⁻⁵</td>
</tr>
<tr>
<td>TEd(90-100ms)</td>
<td>9.6(3, 18)</td>
<td>5.6 ×10⁻⁴</td>
</tr>
<tr>
<td><strong>Hyperpolarising electrotonus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEh(10-20ms)</td>
<td>4.0(3, 18)</td>
<td>2.4 ×10⁻²</td>
</tr>
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<tr>
<td><strong>Recovery cycle</strong></td>
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<td>Superexcitability (%)</td>
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<td>2.9 ×10⁻³</td>
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<tr>
<td>Subexcitability (%)</td>
<td>3.4(3, 18)</td>
<td>4.1 ×10⁻²</td>
</tr>
</tbody>
</table>

**Table 5.1**: Repeated measures ANOVA of rat saphenous nerve nerve excitability parameters comparing carbamazepine 0,3,10 and 30µmol/L. 7 rat nerves studied.
Figure 5.2: Effect of 10\(\mu\)M carbamazepine on 7 rodent saphenous nerves in the skin nerve preparation. Sensory nerve excitability profile plots of a) charge duration, b) 40\% depolarising threshold electrotonus (TE) c) 40\% hyperpolarising TE and d) recovery cycle. Mean values in 7 rodent saphenous nerves are plotted, with no drug present (black line) and with 10\(\mu\)M carbamazepine (red line). Dashed error lines indicate standard error of the mean. Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).
Figure 5.3: Effect of $10 \mu M$ carbamazepine on 7 rodent saphenous nerves in the skin nerve preparation. Sensory nerve excitability profile plots of a) stimulus response b) IV or current threshold curve c) expanded view of the depolarising segment of the IV curve and d) slope of the IV curve. Mean values in 7 rodent saphenous nerves are plotted, with no drug present (black line) and with $10 \mu M$ carbamazepine (CBZ, red line). Dashed error lines indicate standard error of the mean. Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).
Figure 5.4: Effect of 30\(\mu M\) carbamazepine (CBZ) on 7 rodent saphenous nerves in the skin nerve preparation. Sensory nerve excitability profile plots of a) charge duration, b) 40% depolarising threshold electrotonus (TE) c) 40% hyperpolarising TE and d) recovery cycle. Mean values in 7 rodent saphenous nerves are plotted, with no drug present (black line) and with 30\(\mu M\) carbamazepine (orange line). Dashed error lines indicate standard error of the mean. Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).
5.2 The effects of Carbamazepine-Epoxide on rodent nerve ex-vivo

5 individual adult Sprague-Dawley rat saphenous nerves were prepared and increasing doses of carbamazepine-epoxide applied to the portion of nerve in the stimulating chamber as described in section 3.1.

5.2.1 Carbamazepine-epoxide effect maybe detected in the Nerve Excitability Profile of rodent sensory nerve ex-vivo at high concentrations

By 30 $\mu$mol/L carbamazepine-epoxide changes are apparent in the graphical plot of the NEP as seen in Figure 5.5. These changes are in the strength duration curve, depolarising threshold electrotonus, and in the depolarising section of the IV curve seem qualitatively similar to those seen in humans after administration of carbamazepine for sensory nerve (compare with Figure 4.2b, c and g)). Changes seen in the hyperpolarising threshold electrotonus, however, appear reversed from that seen with carbamazepine in humans (compare with Figure 4.2c). Minimal changes were seen in the NEP when plotted graphically for concentrations 1 $\mu$mol/L, 3 $\mu$mol/L of carbamazepine-epoxide (the range seen in human trial participants) when compared with control recording in SIF (Figure 5.7).

The recovery cycle in rodent nerve ex-vivo differs from human by a much shorter RRP (mean of 1.2ms vs a mean of 3.1ms respectively) and less pronounced subexcitability. In the case of short intervals recorded here data proved hard to accurately measure in part due to stimulus artefact at short durations but possibly also due to drug effect at these short durations with a rapid increase in threshold or interference form stimulus artefact becoming problematic to disentangle. Hence comparisons of RRP and refractoriness are not made here in the absence of reliable data.

Repeated measures ANOVA was performed on the ex-vivo saphenous nerve NEP variables for concentrations of carbamazepine-epoxide up to 30 $\mu$mol/L (Table 5.2). In common with the human sensory results, significant effects were seen in the depolarising threshold electrotonus and superexcitability in the recovery cycle. Unlike the human sensory data significant effects were also seen on the peak response, the hyperpolarising threshold electrotonus (a reverse effect when compared with human carbamazepine data), and no significant effect was seen on the IV slope (despite the graphical impression given in Figure 5.8c) or in control threshold, SDTC or rheobase.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>F Value (degrees freedom)</th>
<th>P value</th>
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</tr>
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<td>TEd(peak)</td>
<td>4.2 (5,20)</td>
<td>$9.4 \times 10^{-3}$</td>
</tr>
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<td>TEd(90-100ms)</td>
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<td>TEd(190-200ms)</td>
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<td>S2 accommodation</td>
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<td>TEh(20-40ms)</td>
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<td><strong>Recovery cycle</strong></td>
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<tr>
<td>Superexcitability (%)</td>
<td>2.8 (5, 20)</td>
<td>$4.5 \times 10^{-2}$</td>
</tr>
</tbody>
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**Table 5.2:** Repeated measures ANOVA of rat saphenous nerve NEP parameters comparing carbamazepine-epoxide 0-30 µmol/L. 5 rat nerves studied.
5.2.2 Carbamazepine-epoxide, at the low concentrations experienced by human nerve, showed limited effects in the rodents sensory nerve ex-vivo

The concentration range we are interested in the effects of, to determine whether carbamazepine-epoxide has a physiological effect in our human trial data, are between 0 and 3 \( \mu \text{mol/L} \) (taking into account protein binding as discussed above).

Post-hoc paired t-tests of the parameters shown to vary over the whole range of carbamazepine-epoxide concentration (0-30 \( \mu \text{mol/L} \)) by repeated measures ANOVA, were therefore compared with post-hoc, pairwise t-tests to investigate whether there were significant effects at the concentration range of interest, between 0 and 3 \( \mu \text{mol/L} \).

**Depolarising threshold electrotonus, including the S2 accommodation, showed no change over low concentrations of carbamazepine-epoxide.** No significant differences were seen in any depolarising threshold electrotonus parameters using paired t-tests against baseline for concentrations of carbamazepine-epoxide of 0.3, 1 \( \mu \text{mol/L} \). This finding provides evidence that the major changes in these parameters seen in the humans study are not due to the small levels of carbamazepine-epoxide present over the testing period but rather the primary drug carbamazepine.

**Recovery cycle superexcitability shows a minor reduction in amplitude by 3 \( \mu \text{mol/L} \) carbamazepine-epoxide but this is not likely to have been the dominant effect on the superexcitability reduction seen in humans.** The superexcitability at low concentrations of carbamazepine-epoxide shows a minor reduction in amplitude from a baseline to 3 \( \mu \text{mol/L} \) carbamazepine-epoxide (p<0.001). This effect was not maintained at higher concentrations of carbamazepine-epoxide (10 and 30 \( \mu \text{mol/L} \)) where the effects on pairwise post-hoc comparison were not significant. As the ANOVA shows an overall effect of carbamazepine-epoxide on superexcitability, it is possible that a minor contribution to the reduction in superexcitability seen in humans maybe due to carbamazepine-epoxide. However as 30 \( \mu \text{mol/L} \) failed to produce a significant change on group comparisons, it is likely that this effect is small and can be accounted for by the effect of carbamazepine itself. As mentioned above, recording of the recovery is technically problematic at short duration in these trials and no comparison is possible for the RRP.

**Peak response showed a significant decrease with 3 \( \mu \text{mol/L} \) carbamazepine-epoxide, no corresponding decrease was seen in the human trial data.** No differences were seen between groups for 0, 0.3 or 1 \( \mu \text{mol/L} \).
carbamazepine-epoxide however at 3\(\mu\)mol/L paired t-test showed a reduction from a mean of 3.46mV at baseline to 2.73mV (p<0.05). This finding may represent a true effect of carbamazepine-epoxide producing a conduction block, however as this effect was not seen in the human nerve the possibility arises that this maybe related to the ex-vivo system. The drug was increased sequentially in time in the ex-vivo system, and it cannot be excluded that time in the recording system may also account for this decrease in peak response for either technical (such as slow change in oil level) or physiological reasons (such as deterioration in health of the nerve).

**Hyperpolarising threshold electrotonus showed significant effects in the reverse direction to those seen in human subjects**  Interestingly there was an effect on the hyperpolarising threshold electrotonus with progressively increasing threshold change present by 0.3\(\mu\)mol/L (p<0.05) and maximal by 30\(\mu\)mol/L (p<0.01)(see Figures 5.7c and 5.5c). The change in humans was in the opposite direction and therefore seems primarily due to carbamazepine itself. As with the peak response a contribution of the ex-vivo system accounting for this change cannot be entirely excluded.

**No changes were seen in the SDTC, rheobase, control threshold or IV curve at low concentrations of carbamazepine-epoxide**  Repeated measures ANOVA failed to show effects of carbamazepine-epoxide on the charge-duration plot, control threshold or IV curve. The effects seen on these parameters in humans, are therefore concluded to be solely due to carbamazepine itself.
**Figure 5.5:** Effect of 30µM carbamazepine-epoxide (CBZe) on 5 rodent saphenous nerves in the skin nerve preparation. Sensory nerve excitability profile plots of a) charge duration, b) 40% depolarising threshold electrotonus (TE) c) 40% hyperpolarising TE and d) recovery cycle. Mean values in 5 rodent saphenous nerves are plotted, with no drug present (black line) and with 30µM carbamazepine-epoxide (red line). Dashed error lines indicate standard error of the mean. Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).
Figure 5.6: Effect of 30µM carbamazepine-epoxide (CBZe) on 5 rodent saphenous nerves in the skin nerve preparation. Sensory nerve excitability profile plots of a) stimulus response b) IV or current threshold curve c) expanded view of the depolarising segment of the IV curve and d) slope of the IV curve. Mean values in 5 rodent saphenous nerves are plotted, with no drug present (black line) and with 30µM carbamazepine-epoxide (red line). Dashed error lines indicate standard error of the mean. Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).
Figure 5.7: Effect of carbamazepine-epoxide (CBZe) at low doses 0-3 $\mu M$ (equivalent to serum level seen in humans) on 5 rodent saphenous nerves in the skin nerve preparation. Sensory nerve excitability profile plots of a) charge duration, b) 40% depolarising threshold electrotonus (TE) c) 40% hyperpolarising TE and d) recovery cycle. Mean values in 5 rodent saphenous nerves are plotted, with no drug present (black line) and with 1$\mu M$ carbamazepine-epoxide (turquoise line) and 3$\mu M$ carbamazepine-epoxide (yellow line). Dashed error lines indicate standard error of the mean. Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).
Figure 5.8: Effect of carbamazepine-epoxide (CBZe) at low doses 0-3 \( \mu M \) (equivalent to serum level seen in humans) on 5 rodent saphenous nerves in the skin nerve preparation. Sensory nerve excitability profile plots of a) stimulus response b) IV or current threshold curve c) expanded view of the depolarising segment of the IV curve and d) slope of the IV curve. Mean values in 5 rodent saphenous nerves are plotted, with no drug present (black line) and with 1\( \mu M \) carbamazepine-epoxide (turquoise line) and 3\( \mu M \) carbamazepine-epoxide (yellow line). Dashed error lines indicate standard error of the mean. Axes are labelled, including units where applicable, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000).
Summary

In this chapter we have seen that, as in the human trial, the effects of carbamazepine maybe detected in the NEP of rodent sensory nerve ex-vivo. On visual comparison striking similarity between the effect of carbamazepine on the depolarising threshold electrotonus on rodent and human sensory nerve is appreciable. In rodent nerve however some of the other prominent changes seen in human such as the changes in the strength duration curve, were not reproduced. It should be noted that standard errors are wider than the results from the skin the preparation in a very narrow standard errors seen in the data from the trial participants. It is likely that more minor changes are not picked up due to this variability in the ex vivo system.

Carbamazepine-epoxide is also shown to have a detectable effect at the higher dose of 30 µM, whereas at the significantly lower concentrations measured in the serum of the trial participants (up to 3.5µM), no significant effects are seen on the NEP, thus supporting Hypothesis 2, that low doses of carbamazepine-epoxide will not produce detectable effects on the NEP but mimicking carbamazepine at higher doses.
Chapter 6

Results - Threshold Tracking with Repetitive Stimulation to Elucidate Frequency-Dependence

The aim of this chapter is to describe the effects of stimulating human and rodent ex-vivo nerve at different frequencies, while measuring the effect this change in frequency, has on their excitability threshold.

It is first noted that due to the effects of the muscle velocity recovery cycle this technique would not be reliable for measuring motor nerve excitability at varied frequencies. Thus the chapter describes the results for human and rodent sensory nerve.

The results are shown for physiologically relevant frequencies between 5 and 20 Hz, and compared to a baseline of 2 Hz.

The chapter then goes on to show the effects of lidocaine, carbamazepine and tetrodotoxin, when applied to the rodent sensory nerve, under the same frequency protocol.

6.1 The effects of frequency stimulation on threshold in human nerve and rodent sensory nerve ex-vivo

The method for threshold tracking at different frequencies is described in section 3.7. Figure 6.1 shows the raw data recorded in the QTRAC application, running the frequency adapted threshold tracking program.
Figure 6.1: Original recording of threshold dependence on frequency of stimulation in single human subject (the author). The figures shows the original data as displayed in the data collection program QTRAC. All channels are plotted against elapsed recording time in minutes. The upper most channel plots the stimulus intensity in mA, this is the tracked threshold to maintain a constant amplitude response. The second channel shows the latency of the recorded response in ms. The third channel plots the peak amplitude of the response in mV, the program attempts to keep this constant and the deviation from desired response is used to adjust the stimulus proportionally i.e. tracking. The final channel indicate the frequency of the applied stimulus measured in Hz.
6.1.1 Threshold tracking during high frequency stimulation changes the morphology of the compound muscle action potential but not sensory nerve action potential

Recordings from sensory nerve are made using electrodes placed over digital sensory nerve whereas for motor nerve stimulation the recording electrodes are placed over muscle. During low frequency stimulation such as used in previous chapters this is a reliable method for measuring nerve threshold. At higher frequencies the compound muscle action potential recorded changes morphology whereas the sensory nerve action potential does not. This phenomenon is illustrated in human nerve in Figure 6.2 where the compound muscle action potential duration is reduced. The phenomenon is most likely explained by considering the effect of the muscle velocity recovery cycle (Z’Graggen and Bostock, 2009) in which after repeated stimuli the muscle conduction velocity increases, this in turn leads to a shortening of the duration of the compound muscle response. In this circumstance baseline to negative peak, as a method assessing the proportion of activated motor nerve fibres, becomes unreliable and hence threshold tracking is not reliable. In the human study, the focus is on sensory nerve where the technique accurately represents threshold. The rodent ex-vivo preparation only measures sensory nerve and hence the morphological change in compound muscle action potential is not relevant.

6.1.2 Threshold increases progressively in both human and rodent sensory nerve with increasing number of stimuli at frequencies as low as 5Hz. More pronounced threshold increase is seen at higher frequencies

Recordings were made in 11 healthy human controls and 19 rodent sensory nerves to assess the effects of threshold tracking at frequencies of 5-20Hz compared with baseline stimulation at 2Hz. After a 3 minute period at 2Hz to establish a baseline threshold, a train of 500 stimuli at the chosen frequency was delivered. After the higher frequency stimulus train, stimulation returned to 2Hz to establish recovery to baseline. The results, expressed as percentage change in threshold, are shown in Figure 6.3.

Paired t-tests against baseline were performed to assess significance of these changes at different numbers of stimuli after commencement of the higher frequency stimulation, this is shown in Figure 6.4.
**Figure 6.2**: Human compound action potential waveforms at 2Hz and 20Hz. Recordings are from a representative recording. 20 waveforms at each frequency (at the end of the 500 stimuli train for 20Hz) were averaged. a) Sensory nerve action potential, the nerve response in μV is plotted against time in ms with 0ms centred on the peak of the response. No change in waveform is seen between 2 and 20Hz stimulation. b) Compound muscle action potential measured in mV again plotted against time in ms. A decreased duration of response is seen at 20Hz in comparison with 2Hz stimulation.
Results show, that a threshold change is seen after as few as 50 stimuli at 5Hz, and that the threshold progressively increases with increasing numbers of stimuli.

The effects of 10 and 20Hz are similar but more marked, with 20 Hz producing a rapid and increasing threshold. The threshold in human sensory nerve, increases to a mean of 3.6% above baseline at 50 stimuli, and 7.2% above baseline by 500 stimuli (p<0.00001 in both cases). In rodent nerve there is an increase in the threshold to a mean of 2.3% above baseline at 50 stimuli, and 4.4% at 500 stimuli (p<0.00001 in both cases).

6.1.3 Return of threshold to baseline takes minutes indicating a period of prolonged sub excitability after 500 stimuli at frequencies of 5Hz and above

As can be seen from Figure 6.3 return to baseline is not immediate following cessation of higher frequency stimulation. This prolonged increase in threshold following 500 stimuli is seen in both human and rodent nerve at 5Hz and above.
Figure 6.3: Threshold change in a) 11 human sensory nerves and b) 19 rodent sensory nerves on repetitive stimulation. 500 stimuli at 5, 10, 15 and 20 Hz were applied (stimuli number 1-500) after a baseline stimulation of 2Hz. The period of increased frequency stimulus is represented by the black solid horizontal line. In the human study only frequencies of 5, 10 and 20Hz were used. After 500 stimuli, stimulation returns to 2Hz. Threshold change as a percentage of baseline is plotted against stimulus number. Shaded coloured areas indicate standard error of the mean and solid coloured lines indicate mean at respective frequencies. Small fluctuations seen in the plot b) are due to periodic fluctuations of the fluid level in the skin nerve preparation a result of pump action which despite efforts could not be further minimised.
Figure 6.4: Statistical significance of threshold change in a) human and b) rodent sensory nerve on repetitive stimulation. Paired t-tests against baseline for different numbers of stimuli, at different frequencies of stimulation, 5, 10 and 20Hz in 11 human sensory nerves, and 5,10,15 and 20Hz in 19 rodent sensory nerves. Mean threshold change from baseline, expressed as a percentage, is plotted against number of stimuli (as a categorical grouping on the x-axis) with error bars indicating standard error of the mean. Significant difference from baseline (at stimulus -1) is represented above each error bar: NS-Not significant * p<0.05 ** p<0.01 *** p<0.001. P-values used to determine significance were corrected with Bonferroni correction.
6.2 The effects of lidocaine, carbamazepine and tetrodotoxin on rodent nerve ex vivo at different frequencies of stimulation

Recordings showing the effects on the threshold of rodent sensory nerve in the skin nerve preparation of lidocaine Figure 6.5 and Figure 6.6, carbamazepine Figure 6.7 and Figure 6.8, and tetrodotoxin Figure 6.9 and Figure 6.10 at different frequencies between 5 and 20Hz and at different concentrations.

6.2.1 Lidocaine produces a measurable frequency dependent inhibition in myelinated rodent sensory nerve at stimulation frequencies between 5 and 20Hz.

Figure 6.5 shows the mean effect on threshold change of concentrations of Lidocaine ranging from 0-100µM. Figure 6.6 shows that compared with no drug, when frequencies of 5Hz and above are applied to the nerve, Lidocaine produces a significant increase in threshold after as few as 50 stimuli at concentrations of 30 and 100µM. No consistent effect is seen from a concentration of 10µM.

6.2.2 Carbamazepine shows no frequency dependent inhibition in myelinated rodent sensory nerve at stimulation frequencies between 5 and 20Hz and concentrations up to 30µM. However at 5Hz stimulation carbamazepine produces a facilitation or relative lowering of threshold.

Figure 6.7 shows the mean effect on threshold change of concentrations of carbamazepine ranging from 0-30µM. Figure 6.8 shows that compared with no drug, carbamazepine does not produce a relative change in threshold at frequencies between 10 and 20Hz. At 5Hz there is a facilitation or relative reduction in threshold at drug concentrations between 3 and 30 µM.
Figure 6.5: Effect of lidocaine on threshold at concentrations from 0-100 µM in 6 rodent saphenous nerves. The coloured key indicates the concentrations of lidocaine. Mean threshold change as a percentage of baseline threshold at 2Hz is plotted against stimulus number. A train of 500 stimuli starting at stimulus number 1 is given at 5,10,15 or 20Hz (which are plotted sequentially in the upper to lower plots). The black horizontal bar indicates the period of increased frequency stimulation before reverting back to a 2Hz baseline.
Figure 6.6: Effect of Lidocaine on threshold at concentrations from 0-100µM in 6 rodent saphenous nerves. Mean threshold change as a percentage of non-drug treated nerve threshold (control) is plotted for drug concentrations tested. This effect of drug on threshold is then shown for the range of frequencies and number of stimuli tested. Un-paired t-tests against a control of 0µM are plotted for concentrations of drug recorded. Means are plotted with error bars indicating standard error of the mean. Significant difference is represented above each error bar: NS-Not significant * p<0.05 ** p<0.01 *** p<0.001 **** p<0.0001 ***** p<0.00001. P-values used to determine significance were corrected with Bonferroni correction.
Figure 6.7: Effect of carbamazepine on threshold at concentrations from 0-30µM in 5 rodent saphenous nerves. The coloured key indicates the concentrations of carbamazepine. Mean threshold change as a percentage of baseline threshold at 2Hz is plotted against stimulus number. A train of 500 stimuli starting at stimulus number 1 is given at 5, 10, 15 or 20Hz (which are plotted sequentially in the upper to lower plots). The black horizontal bar indicates the period of increased frequency stimulation before reverting back to a 2Hz baseline.
Figure 6.8: Effect of carbamazepine on threshold at concentrations from 0-30µM in 5 rodent saphenous nerves. Mean threshold change as a percentage of non-drug treated nerve threshold (control) is plotted for drug concentrations tested. This effect of drug on threshold is then shown for the range of frequencies and number of stimuli tested. Un-paired t-tests against a control of 0µM are plotted for concentrations of drug recorded. Means are plotted with error bars indicating standard error of the mean. Significant difference is represented above each error bar: NS-Not significant * p<0.05 ** p<0.01 *** p<0.001 **** p<0.0001 ***** p<0.00001. P-values used to determine significance were corrected with Bonferroni correction.
6.2.3 Tetrodotoxin shows frequency dependent facilitation between 5 and 20Hz, at concentrations up to 5nM in myelinated rodent sensory nerve.

Figure 6.9 shows the mean effect on threshold change of concentrations of Tetrodotoxin ranging from 0-5nM. Figure 6.10 shows that compared with no drug, Tetrodotoxin lowers threshold at stimulation frequencies between 5 and 20Hz at concentrations up to 5nM.

Figure 6.11 compares the effects of carbamazepine, lidocaine and tetrodotoxin at 10Hz stimulation compared with baseline 2Hz at differing numbers of stimuli at 10Hz and drug concentration to enable a comparison. It can be seen that only lidocaine produces a significant frequency dependent increase in relative threshold at 10Hz which starts as early as 50 stimuli. No increase in threshold is seen with either carbamazepine or tetrodotoxin at 10Hz up to 500 stimuli.
Figure 6.9: Effect of tetrodotoxin on threshold at concentrations from 0-5nM in 4 rodent saphenous nerves. The coloured key indicates the concentrations of tetrodotoxin. Mean threshold change as a percentage of baseline threshold at 2Hz is plotted against stimulus number. A train of 500 stimuli starting at stimulus number 1 is given at 5,10,15 or 20Hz (which are plotted sequentially in the upper to lower plots). The black horizontal bar indicates the period of increased frequency stimulation before reverting back to a 2Hz baseline.
Figure 6.10: Effect of Tetrodotoxin on threshold at concentrations from 0-5nM (expressed in figure as µM) in 4 rodent saphenous nerves. Mean threshold change as a percentage of non-drug treated nerve threshold (control) is plotted for drug concentrations tested. This effect of drug on threshold is then shown for the range of frequencies and number of stimuli tested. Un-paired t-tests against a control of 0nM are plotted for concentrations of drug recorded. Means are plotted with error bars indicating standard error of the mean. Significant difference is represented above each error bar: NS-Not significant * p<0.05 ** p<0.01 *** p<0.001 **** p<0.0001 ***** p<0.00001. P-values used to determine significance were corrected with Bonferroni correction.
Comparison of Carbamazepine, Lidocaine and Tetrodotoxin at 10Hz stimulation

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Concentration Change compared with control (%)

Comparison of Carbamazepine, Lidocaine and Tetrodotoxin at 10Hz stimulation

Figure 6.11: Comparison of lidocaine, carbamazepine and tetrodotoxin at 10Hz stimulation. Mean threshold change as a percentage of non-drug treated nerve threshold (control) is plotted for drug concentrations tested. This effect of drug on threshold is then shown for the range of frequencies and number of stimuli tested. Un-paired t-tests against a control of 0nM are plotted for concentrations of drug recorded. Means are plotted with error bars indicating standard error of the mean. Significant difference is represented above each error bar: NS-Not significant * p<0.05 ** p<0.01 *** p<0.001. P-values used to determine significance were corrected with Bonferroni correction.
Summary

This chapter has shown that with stimulation at frequencies as low as 5 Hz, over the course of 500 stimuli, a gradual increase in threshold can be seen for both human and rodent ex-vivo sensory nerve. This supports Hypothesis 3. Also apparent from the results is, that after 500 stimuli, the threshold does not return immediately to baseline.

The addition of lidocaine, carbamazepine and tetrodotoxin to the rodent sensory nerve, under the same frequency protocol, shows variable results. Lidocaine elicits a significant frequency dependent increase in threshold over and above that seen with higher frequencies alone. Carbamazepine shows very little effect on threshold over the protocol and tetrodotoxin confusingly displays a minor reduction threshold compared to control. Hypothesis 4 is partially supported by this data in that lidocaine results confirmed our expectations, however both carbamazepine and tetrodotoxin show somewhat unexpected findings.
Chapter 7

Discussion of Results of Experimental Data

7.1 Discussion

This study has documented the effects of carbamazepine on human peripheral axons in-vivo and shown that, at a therapeutically relevant concentration, the action of this drug can be detected with 100% sensitivity. Also reported are the effects of carbamazepine’s active metabolite, carbamazepine-epoxide, in a rat sensory nerve model. There are comparable studies investigating the effects of sodium channel active compounds on the human NEP. Kiernan et al. (2005a) studied the effects of tetrodotoxin in people accidentally poisoned but whom survived; although some similarities are seen there are qualitative and quantitative differences. Kuwabara et al. (2005) and Isose et al. (2010) studied the effects of the oral lidocaine analogue mexiletine on the NEP in patients with neuropathic pain, the results of which are different to the findings of this investigation. A strength of this study is to investigate normal human nerve. This contrasts with the study patients with neuropathic conditions on a long term medication in whom controlling for underling peripheral nerve abnormality and medication levels is considerably more challenging. The main findings and inferences derived from them of the present study will be discussed and then the differences and similarities to tetrodotoxin and mexiletine will be highlighted.
7.1.1 The effects of carbamazepine on human nerve

Carbamazepine produces significant measurable effect on the axonal membrane at rest in human sensory nerve but not in motor nerve

Perhaps the simplest measure of excitability is the absolute threshold of an axon. In our human participants, carbamazepine increased the threshold needed for excitation of sensory nerve, but had no effect on motor nerve threshold. In both cases the peak response from the nerve was unchanged indicating that carbamazepine did not induce a “conduction block”. The increased threshold in sensory nerve, together with a reduced SDTC, increased rheobase and resting IV slope, provides strong evidence that the drug is affecting ion channels whilst the sensory axon is at its resting membrane potential.

In motor fibres, along with no change in threshold or peak, no significant effect is seen on the SDTC or rheobase. The resting IV slope does however show a small increase. Were carbamazepine having a major effect on the ion channels in motor axons at resting membrane potential, one would expect to see an effect on all these parameters and thus is seems likely that carbamazepine does not have a significant effect on sodium channels in motor axons at rest.

The minor change in resting IV slope however does not fit with this explanation, it may indicate a much more minor affect on channels with resting IV slope being the most sensitive indicator. Another possibility is that the resting IV slope in the protocol used does not have sufficient detail to i.e. too large current steps near rest, to produce a very accurate representation of the resting membrane conductance.

A solution to this would be to repeat the IV curve with much smaller changes around zero current. To minimise the potential affect from time dependent binding of carbamazepine to inactivated states of the channel, shorter conditioning pulses would also provide a possibility of investigating in more detail the resting conductance.

The largest effect of carbamazepine in human peripheral nerve is to reduce excitability in situations of prolonged depolarisation

The largest effects of carbamazepine in humans was seen on the depolarising threshold electrotonus for both motor and sensory nerve. The meaning of this finding is that the nerve is most susceptible to the effects of carbamazepine when it is in a depolarised state. The direction of this effect, as measured by the NEP, is to reduce excitability over the course of a depolarisation. This voltage-dependence of inhibition is well described for carbamazepine in patch clamp and whole nerve voltage clamp recordings (see section 1.7.4) but this represents the first time
The effects seen on motor nerve, indicate that carbamazepine’s effect started within 20ms of initiation of the sub-threshold depolarising pulse, as evidenced by the reduction in TEd(peak). It also must occur within 5ms after an action potential, as demonstrated by the change in superexcitability by this time point.

It is inferred from this that either the motor nerve sodium channels must have a drug bound state at rest, and therefore that a proportion of sodium channels in the motor nerve must be in their inactivated state at rest, or that the drug may bind to a significant proportion of channels within 5ms of an action potential or within 20ms after sub-threshold depolarisations.

Given the lack of changes in typical resting axonal parameters discussed above (absolute threshold, SDTC and rheobase), the most likely explanation appears to be that carbamazepine requires less than 5ms to bind to channels in their inactivated state.

In contrast however, the sensory nerve shows no change in TEd(peak), but an equivalent change at later time points during the depolarising pulse (the earliest effect being at approximately 40ms) to the motor nerve, in which a reduction in excitability is seen. From this one may infer that either the drug only affects a significant portion of the sodium channels after the nerve has been in a depolarised state for a period of time not less than 20ms, and likely starting around 40ms when the first effect is seen, or early changes in depolarising threshold electrotonus are cancelled out by another equal and opposite change, the time course of which is less than 40ms. Again, given the fact that changes are seen in the excitability markers of the resting sensory axon SDTC, rheobase and resting IV slope it appears likely that there is a process present in the sensory nerve which masks the changes in the early portion of a depolarising pulse by acting in the opposite direction. A candidate mechanism for this would be a slight hyperpolarisation of the resting nerve as a result of carbamazepine.

**Carbamazepine use-dependence likely explains the limited effects seen on the recovery cycle**

Refractoriness and RRP are traditionally considered a measure of how fast sodium channels are able to recover from their inactivated state Burke et al. (1998). It is perhaps surprising, given that carbamazepine is thought to bind and prolong recovery from the inactivated state, that no increase is seen in refractoriness or RRP after carbamazepine, and

this finding has been shown electrophysiologically, in-vivo, in humans. The clinical anticonvulsant effect of carbamazepine may at least in part be due to this action on prolonged depolarisations, as paroxysmal depolarising shifts, are thought to be intrinsic to the epileptogenic mechanism (Johnston and Brown, 1984; Rogawski and Löscher, 2004).
if anything there is a very slight reduction in RRP in motor axons. The explanation is likely to be that a single action potential does not present enough opportunity for carbamazepine to bind and produce effects on the refractory period. Multiple action potentials are likely to be needed to see a build up of carbamazepine binding, and were the recovery cycle to be repeated with multiple supra-maximal conditioning pulses it is hypothesised that an effect would be seen. This is essentially the basis of the “use-dependence” reported for carbamazepine (Macdonald and Kelly, 1995). This is also the reasoning behind creating the protocol for threshold tracking at higher frequencies.

The superexcitability following the refractory period, mediated by the depolarising after-potential, is reduced after carbamazepine. The complexity of this relationship between the inter nodally generated depolarising after-potential, sodium channel inactivation and the superexcitability makes providing explanation for this observation challenging. The suggestion is that carbamazepine acts to decrease the depolarising after-potential, possibly by affecting internodal sodium channels.

**Reasons for the difference between motor and sensory nerve**

A possible explanation for the difference between motor and sensory axons lies in their resting membrane potential, with human sensory axons recently being proposed to be at a more depolarised state at rest than motor axons (-80.3mV and -84.4mV respectively) (Howells et al., 2012).

The NaV1.6 sodium channel which produces a fast inactivating, tetrodotoxin-sensitive sodium current, is thought to be the preponderant sodium channel at nodes of Ranvier in peripheral nerve (Caldwell et al., 2000; Krzemien et al., 2000).

Burbidge et al. (2002) found that for the human NaV 1.6, channel a V1/2 of fast and slow inactivation of -53.4mV and -71.6mV respectively (slope factors of 11.6 and 6.5 respectively) from patch clamping of recombinant human channel in HEK293 cells. From this is can be calculated that at the resting membrane potentials of sensory nerve 9% of channels would be in the fast inactivated state and 21% in the slow inactivated state, a total of 30% of channels in inactivated states, and for motor nerve 6% and 12% respectively making a total of 18% in inactivated states. No channels are in the open state at rest for either motor or sensory axon resting potentials.

Wittmack et al. (2004) have demonstrated that a member of the FGF homologous factors (FHF) FHF2B co-localises with NaV 1.6 in dorsal root ganglions and on both unmyelinated and myelinated peripheral sensory nerve fibres, where it localises to the nodes of Ranvier, but is not seen not on peripheral motor nerves. They have shown that FHF2B modulates NaV1.6 channel properties resulting in a 4mV depolarising shift of voltage-dependent inactivation and an increase in the peak cur-
rent. This is an alternative explanation for the differences in excitability between motor and sensory fibres to that of Howells et al. (2012) which has not yet been fully explored.

The increase in S2 accommodation seen in sensory nerve is unlikely to be due to a slow potassium current in this study

It is notable that perhaps the most significant effect of all in our results, and that hypothesised in advance, is the large increase in the S2 accommodation seen in the sensory nerve. This is in line with the findings of Shields and Koltzenburg (2010) in rat nerve and underlines the translational validity of the NEP approach. The S2 accommodation is classically associated with the activity of the slow potassium channels located mainly at the node which act to reduce excitability over long depolarisations due to their slow activation kinetics (Bostock and Baker, 1988; Bostock et al., 1998). It may be thought that an increase in this S2 accommodation seen after carbamazepine in sensory nerve maybe attributable to an increase in the delayed rectifier potassium current through these slow channels, this it is argued, is unlikely. Firstly the same effect is not seen in motor nerve (in fact a reduction in S2 accommodation is seen). Secondly the known state-dependent binding to the inactivated state of the sodium channel can explain the observation, if time is allowed for the drug to bind to this state. Thirdly, although reports are somewhat contradictory (for a review see Ambrósio et al. (2002)), no effect on the Ca\(^{2+}\)-activated or A-type K\(^{+}\) currents were seen after carbamazepine and a reduction in the delayed rectifier K\(^{+}\) current was seen with doses of 100\(\mu\)mol/L (Matsumoto et al., 1998), this dose is well above the level seen in our subjects and also in the wrong direction to explain the results we see.

7.1.2 The effects of carbamazepine and carbamazepine-epoxide in rat nerve

The findings of Shields and Koltzenburg (2010) of the effects of carbamazepine in the rat ex-vivo sensory nerve show similar effects on the charge-duration plot, the IV curve and depolarising threshold electrotonus. The recovery cycle shows reductions in superexcitability as seen in the human study however the RRP appears to increase in the rat nerve which may be a result of the higher concentration achieved (up to 100\(\mu\)mol/L). The only major difference appears in hyperpolarising threshold electrotonus which increases in amplitude in the rat nerve, a finding which was also seen in the carbamazepine-epoxide rat nerve experiments presented here. This finding maybe a result of the ex-vivo setup as discussed in section 5.2.2 however this finding remains at present

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remains unexplained. The high degree of similarity between the results obtained in the rat nerve ex-vivo and the human study demonstrate convincingly the translatable nature of the NEP.

Human nerves experienced concentrations up to $3.5\mu\text{mol/L}$ of carbamazepine-epoxide. Taking into account protein binding, the true concentration experienced is likely $<2\mu\text{mol/L}$. Section 5 provides evidence that, at these low levels, carbamazepine-epoxide has no effect on rat sensory nerve ex-vivo. These low concentrations of carbamazepine-epoxide showed no effect on the rat resting membrane NEP parameters in contrast to the changes seen in the human sensory study.

It is thus argued that the main effects seen in the human study on the NEP parameters was from carbamazepine itself rather than the metabolite.

Carbamazepine-epoxide at high concentrations (not seen in our participants) did produce significant effects on the NEP in the rat ex-vivo model confirming that as previously found this metabolite is an active compound (Bertilsson and Tomson, 1986). This study provides further evidence that, at concentrations between 10 and $30\mu\text{mol/L}$, it is likely to have a physiologically relevant effect in patients where similar concentrations occur with prolonged use.

### 7.1.3 Comparison of carbamazepine with tetrodotoxin and mexiletine

**Comparisons with tetrodotoxin effects on the nerve excitability profile**

It is necessary to compare the results obtained here with those obtained looking at other sodium channel active compounds whose effects have been studied with the same technique in human peripheral nerve in-vivo. Kiernan et al. (2005a) studied the effects of tetrodotoxin in-vivo in humans accidentally poisoned. The findings differ from those reported here.

After tetrodotoxin, motor nerve changes were seen in parameters of the resting axon state (SDTC, rheobase, threshold) whereas we find no change in these parameters after carbamazepine. Depolarising threshold electronic changes were very similar to those seen here with reductions in excitability throughout the depolarising pulse. Hyperpolarising threshold electrotonus changes, however, were in the opposite direction to those we see. RRPs and superexcitability decreased in both groups, however late subexcitability was reduced after tetrodotoxin but not after carbamazepine.

Sensory nerve changes after tetrodotoxin were essentially the same as the motor nerve (with some quantitative differences), with the ex-
ception that depolarising threshold electrotonus did not change significantly. After carbamazepine the sensory findings are similar to those with tetrodotoxin, with the exception that depolarising threshold electrotonus showed the greatest change of all in our study, and no change in subexcitability is seen.

Mathematical modelling using the myelinated nerve model described in section 1.6.1, produced an excellent fit for the findings after tetrodotoxin with a reduction in Na\(^+\) permeability by a factor of 2 (Kiernan et al., 2005a). This reduction changed the relationship between membrane potential and threshold and explains the effects seen in the NEP parameters excellently. Many of the observed effects of tetrodotoxin were counterintuitive to the authors and only elucidated by the application of modelling. The effects of mathematical modelling of the results presented will be discussed further in chapter 8.

**Comparisons with mexiletine effects on the nerve excitability profile**

Isose et al. (2010) and Kuwabara et al. (2005) have assessed the sodium channel blocker mexiletine in patients with neuropathic pain and severe muscle cramping. These authors did find motor axon changes in SDTC, rheobase and refractoriness, along with sensory axon changes in SDTC and latent addition, but not in others markers of excitability. The lack of changes in other parameters was unusual. The study was complicated by the participants likely having abnormal nerves, and only a moderate dose of mexiletine being used without knowledge of the serum level. Although carbamazepine also seems to affect the SDTC in sensory axons, it had no effect on the SDTC of motor axons unlike mexiletine. Carbamazepine notably produces changes on threshold electrotonus which, unusually, were not seen with mexiletine.

**7.1.4 Summary**

It has been demonstrated for the first time in healthy human nerve in-vivo that carbamazepine has a pharmacological action on peripheral myelinated sensory and motor nerve excitability, the nature of this action has been described. Previously this has been inferred from experimentation on nerve preparations outside of their physiological environment or in animal models. The hypotheses set out at the start (see section 2.2) of this thesis have been successfully demonstrated.

The conclusion that is drawn, is that the NEP is a sensitive test to detect the effects on peripheral nerve in healthy human volunteers of an ion channel active medication, in this case carbamazepine. The findings have been related to specific known mechanisms of action of car-
bamazepine and the NEP as being able to demonstrate “target engagement” of the drug in human peripheral nerve in-vivo. The comparison with NEP findings of carbamazepine-epoxide and carbamazepine effect in the rat nerve demonstrate the translatable nature of the NEP.

These findings together, lead the author to propose the NEP as a translatable biomarker suitable for investigation of the mechanism of action and demonstration of target engagement for compounds active on the human peripheral nerve sodium channels.

Limitations of the technique are that it is only suitable for assessment of large myelinated peripheral nerve fibres rather than thinly or unmyelinated fibres. There remains scope for improving the interpretation of the findings of this technique in the context of state dependent compounds through adaptation of the mathematical model for myelinated nerve to incorporate drug effect, this is the focus of chapter 8.

### 7.1.5 The effects and measurability of frequency in normal myelinated nerve

In order to assess use-dependent sodium channel blockers, a mechanism of measuring axonal excitability during, as well as after, nerve stimulation at higher frequencies is required. The present study demonstrates that in non-drug treated, healthy human and rodent ex-vivo sensory nerve, threshold tracking is a reliable method for this but is problematic in motor nerve.

The study shows that there is a frequency-dependent increase in axonal threshold elicited by prolonged stimulation of up to 500 stimuli at frequencies of 5Hz and above. This change in normal nerve, it is argued, reflects hyper-polarisation of the axonal membrane as a result of both accumulation of the sub-excitability of the recovery cycle (Bergman’s H1) and the electrogenic sodium pump activity (Bergman’s H2).

Two types of sub-excitability following nerve activity are described by Bergmans (1970) following trains of impulses. The first is the H1 period or sub-excitability of the recovery cycle, and is mediated by slow K\(^+\) channels, being blocked by tetraethylammonium (Baker et al., 1987). Slow K\(^+\) channels, which are activated following an action potential, are slow to deactivate. This results in a period of hyper-polarisation following the early sub-excitability, and the increase in threshold seen in the recovery cycle (Baker et al., 1987; Lin et al., 2000). This sub-excitability in human axons, reaches a peak after 7-10 impulses at high frequency e.g. 250Hz and is over within 100-200ms (Bergmans, 1970; Burke et al., 2001).

The period of sub-excitability lasting seconds to minutes, or H2 period, develops after more prolonged stimulation, and is mediated by the hyper-polarisation produced by activity of the sodium pump. Bostock and
Grafe (1985) showed that replacement of NaCl with LiCl (which is able to replace Na$^+$ as a charge carrier, but does not stimulate the Na$^+$K$^+$pump) abolished the H2 period. The stimulus frequency and duration, required to elicit the H2 sub-excitability, according to Bergmans (2012) is somewhere between 1200 and 1800 stimuli at a frequency of between 10 and 15Hz.

Threshold tracking at higher frequencies is a reliable measure of axonal excitability recording from sensory nerve but not in motor nerve recording from muscle

Figure 6.2 shows that threshold tracking of motor nerves, using the muscle response, problematic due to the change in its waveform. This change in waveform also supports Bergmans (2012) criticism of the findings of Noto et al. (2011), that muscle fibre latency is not a reliable measure of axonal threshold at higher frequencies. A mechanism to overcome this limitation would be to use threshold tracking of single motor units at higher frequency rather than a latency measurement, this has so far not been achieved by the author.

Noto et al. (2011) measured latency as a surrogate marker of axonal threshold in human motor nerve. They found that, with progressive numbers of stimuli at frequencies between 10 and 20Hz, the latency of the recorded muscle fibre action potential gradually prolonged. This was interpreted by the authors as indicating an increase in axonal threshold, due to the sodium pump or an H2 period, and elicited within 500 stimuli. This conclusion was questioned by an accompanying editorial by Bergmans (2012), which raised the question of the role of the muscle fibre recovery velocity function in the study, and the ability to elicit H2 with such a short number of stimuli.

Here, therefore, threshold tracking of sensory nerve only is used to assess excitability changes in normal nerve and with drug.

Threshold tracking of sensory nerve at frequencies of 5Hz-20Hz shows comparable results when measured in human nerve in-vivo and rodent nerve ex-vivo, and is thus a translatable measure.

Figure 6.3 demonstrates the translatability of the technique between human and rodent nerve preparations. The results show a similar overall pattern, despite the maximum threshold increase in human nerve being higher than in the rodent ex-vivo nerve. It is not known, whether this difference represents a species difference, a difference between the specific nerves recorded i.e. median vs saphenous, or between the preparations used in-vivo vs ex-vivo.
Threshold tracking of sensory nerve at frequencies of 5Hz-20Hz elicits the H1 sub-excitability accounting for an early increase in threshold (by 50 stimuli) and also the H2 sub-excitability accounting for a progressive rise in threshold and delayed return to baseline (between 50-500 stimuli).

Figure 6.3 and Figure 6.4 show that, at frequencies of 5Hz and above, there is a progressive increase in threshold between 50 and 500 stimuli. The H1 period is thought to plateau after 10 stimuli and hence cannot account for this progressive increase. Similarly at the offset of higher frequency stimulation, return to baseline takes minutes rather than being over within 200ms which maybe expected if H1 were to account for the changes seen. At 5Hz, a buildup of H1 would not be expected as the inter-stimulus interval is 200ms which is outside of the period relevant to H1.

At 10, 15 and 20Hz however the inter stimulus interval approaches and enters the period where H1 build up becomes relevant, and hence a contribution of H1 may at least partly explain the early rise in threshold at stimulus numbers up to 10. Again however, the progressive increase in threshold, and delay in return to baseline, are characteristics of the H2 period. The H2 period however is not thought to be elicited until after many more stimuli, and thus the large changes in threshold seen by 50 stimuli are likely largely accounted for by the H1. This is at odds with the assertion of Bergmans (2012) that the H2 is only elicited after much higher numbers of stimuli, and more in keeping with the findings of Noto et al. (2011) despite the possible limitations of their study. It should also be noted that these findings are for sensory nerve whereas the previously reported studies are for motor nerve. It maybe that the H2 is elicited earlier in sensory nerve than motor nerve.

It is therefore proposed that the mechanism accounting for the increase in threshold seen early in the train is likely to be the H1 with the buildup of slow $K^+$ channel activation, but the mechanisms accounting for the further progressive rise, seen between 50 and 500 stimuli, being the H2 mediated by activity of the pump.

7.1.6 Use-dependence of sodium channel blockers measured with threshold tracking in human and rodent sensory nerve

Having established the response of normal sensory nerve, as measured with threshold tracking, to trains of stimuli, the effect of classic sodium channel blocking medication lidocaine, carbamazepine and tetrodotoxin were assessed. Their effects were expressed as a percentage change.
from the normal response to the frequency protocol, Figures 6.6 to 6.10.

According to the classification of Na\textsuperscript{+} channel blockers described by Lenkey et al. (2010) and discussed in 2.3.2, lidocaine and carbamazepine are classified as type 2 blockers, with low potency, fast kinetics and almost full reversibility. Lidocaine is distinguished from carbamazepine in their study, by showing use-dependence whereas carbamazepine does not (5Hz stimulation used), thus lidocaine is labelled as type 2a and carbamazepine as type 2b. Tetrodotoxin was not measured in the study but is likely to fit into category 3 drugs, those with high potency, apparent irreversibility and no use-dependence. It should be noted that the term use-dependent to describe a sodium channel blocker, refers to use-dependent inhibition and hence drugs showing use-dependent facilitation are not classically referred to as use-dependent.

Threshold tracking at higher frequencies is a sensitive measure for sodium channel blocker use-dependent inhibition in rodent sensory nerve ex-vivo, and these findings are expected to be translatable to humans.

As discussed in section 6.2.1, the results of this study show that lidocaine shows use-dependent inhibition at frequencies of 5Hz and above, carbamazepine shows no use-dependent inhibition but if anything a mild facilitation at 5Hz and that tetrodotoxin shows a use-dependent facilitation between 5 and 20Hz. Of note carbamazepine’s measured value representing use-dependence according to Lenkey et al. (2010) was 0.98. A value below 1 in their study although not explicitly stated may represent a use-dependent facilitation of excitability which is of interest given the slight facilitation also found in our study with carbamazepine.

Some authors have found evidence of use-dependent inhibition by carbamazepine at 10Hz in cultured rat cortical neurons (Errington et al., 2008), and at 10Hz in rat myelinated peripheral nerve (Schwarz and Gogat, 1989) and also in bullfrog nerve (Courtney and Etter, 1983).

These finding are at odds with the results of Lenkey et al. (2010) and also the results presented here. A possible explanation arises from the resting potential used in the voltage clamp protocols which, if artificially depolarised may induce features of use-dependence not seen at more hyperpolarised potentials. At higher frequencies of 100Hz and above there is evidence of use-dependence of inhibition which is not surprising (McLean and Macdonald, 1986) and thus when discussing use-dependence it is important to note the conditions and frequency which are being referred to. This point highlights Hille’s concern of variation in attributed properties of a drug from laboratory to laboratory given the intricacies of the mechanisms involved.

The results in this chapter thus separate the three sodium channel blockers lidocaine, carbamazepine and tetrodotoxin according to their
use-dependent properties in rodent sensory nerve ex-vivo. That the results from the rat nerve without drug are similar to humans as discussed in section 7.1.5, means one may predict that this technique, of threshold tracking at varying frequencies, represents a translatable biomarker of use-dependence applicable to human and rodent nerve. This property makes it a potentially valuable tool in assessing characteristics of sodium channel blocking agents in humans to complement the traditional nerve excitability profile discussed in chapters 4 and 8.

7.1.7 Summary and Conclusions

A postulated limitation of the standard nerve excitability profile is the lack of sensitivity for use-dependence. This study has developed a technique of threshold tracking at higher frequencies, and demonstrated validity of the technique in rodent and human sensory but not motor nerve.

This novel technique, it has been argued, demonstrates the build up of recovery cycle sub-excitability responsible for the H1 period, as well as the activity of the sodium pump responsible for the H2 period of sub-excitability, and that it is translatable between human in-vivo nerve and rodent ex-vivo nerve.

The technique has demonstrated the use-dependent characteristics of lidocaine, the relative non use-dependence of carbamazepine at frequencies up to 20Hz, which is found to be in agreement with previously published measures.

The two hypotheses that: “repetitive stimulation of myelinated nerve will increase threshold of the nerve in a frequency dependent manner and will be measurable by the technique of threshold tracking”, and that: “the use dependent characteristics of three different sodium channel blockers; lidocaine, carbamazepine and tetrodotoxin will be quantifiable using the repetitive stimulation and threshold tracking in the rodent skin-nerve preparation”, have been confirmed.
Chapter 8

Modelling of Sodium Channel Inhibition in Myelinated Nerve

8.1 Background and Hypothesis

8.1.1 Application of modelling to electrophysiology

Mathematical modelling as a method of describing the nerve impulses has a long history as discussed in chapter 1 sections 1.6 and 1.6.1. The most famous success is that of the original work by Hodgkin and Huxley (1952b,a,c,d), in which they provided an elegant mathematical description of the nerve action potential which is still in use today. It was this work that earned Hodgkin and Huxley the Nobel Prize in Physiology or Medicine in 1963.

The strength of the modern nerve excitability profile, is its basis in a mathematical model described by Bostock et al. (1991), and shown in Figure 1.10. The Bostock model built upon the insight and model of Barrett and Barrett (1982) as well as utilising the equations of Hodgkin and Huxley (1952d). The addition of persistent sodium conductance to the model by Bostock and Rothwell (1997) made a further step in describing the conductances responsible for axonal excitability in myelinated fibres.

The Bostock model as a method of analysing the nerve excitability profile has been shown for example to be sensitive to detect the action of tetrodotoxin on human nerve (Kiernan et al., 2005a) as well as the Kv1.1(KCNA1) fast potassium channel mutation in patients with episodic ataxia type 1 (Tomlinson et al., 2010).

More recently the Bostock model has been adapted in order to explain differences seen in excitability between human motor and sensory fibres, with a slight depolarisation in the voltage dependence of Ih and a reduced nodal slow potassium conductance in sensory fibresHowells et al. (2012).
8.1.2 Modelling of sodium channel inhibiting drugs

The therapeutic action of sodium channel inhibiting drugs is thought to be related to their mechanism of action on the sodium channel and specificity for particular sodium channel subtype Zuliani et al. (2009); Catterall (2012); Levinson et al. (2012); Kuo et al. (1997). Sodium channel blockers are being actively developed by drug companies as potential therapeutic agents for various conditions including epilepsy, migraine and neuropathic pain. The limitation of test systems and lack of availability of biomarkers has been cited as a barrier to development Zuliani et al. (2009); Chizh and Sang (2009); Frank and Hargreaves (2003). In previous chapters it has been shown that the nerve excitability profile along with threshold tracking at higher frequencies provide a sensitive translatable biomarker for sodium channel blockers.

Karoly et al. (2010) have applied mathematical modelling to describe the action of sodium channel blockers in a patch clamp system. The authors presuppose that for sodium channel blockers, “the major mechanism of inhibition is preferential affinity to, and stabilization of a specific inactivated state” and that concern regarding the exact site of binding is not relevant to the modelling. Two types of model were used by the authors, 1) a classical but modified Hodgkin and Huxley model and 2) a multistep activation model or Markov Chain type model. The models were based on the modulated receptor hypothesis of Hille (1977a) discussed in chapter 6. Sodium channel blockers in this paper were considered as either preferentially affecting the fast or slow inactivated state of the sodium channel, and further considered to either have fast or slow binding kinetics. Classic anticonvulsants e.g. carbamazepine were considered to have a fast inactivated state binding preference and fast kinetics, with parameters matched to experimental data such as Kuo et al. (1997). The results confirmed with modelling, the fast inactivated state preference and fast binding dynamics of the classic anticonvulsants carbamazepine, phenytoin and the anaesthetic lidocaine. The results also importantly noted the problem of classic voltage protocols, used to assess drug preference to the slow inactivated state.

8.1.3 Adapting current models

The aim of this chapter is to adapt the Bostock model to be able to account for effects of a sodium channel blocker and describe its state dependent activity. The model described by Karoly et al. (2010) is used as a basis for this. If this is achievable and nerve excitability results derived from human drug studies are accountable by a mathematical model of drug effect, the technique becomes valuable as not only a biomarker of target engagement but as a tool to investigate mechanistic effect in the living human system.
8.2 Methods

The method to apply modelling of sodium channel inhibiting compounds used in this chapter is to utilise the current validated Bostock model and introduce into this model variables allowing simulation of drug effect as described by Karoly et al. (2010).

8.2.1 The Bostock Model

In order to adapt the model, the current model must be stated in full. The model is as first fully described in Bostock et al. (1991), updated to include persistent sodium conductance by Bostock and Rothwell (1997), and re-described and fitted to sensory human sensory nerve by Howells et al. (2012). The model is illustrated in figure 8.1:

\[
\frac{dE}{dt} = \frac{- (I_{Na} + I_{Kf} + I_{Ks} + I_{Lk} + I_{pump} + I_{external} + I_{BB})}{(C_n + C_{myelin})} \tag{8.1}
\]

\[
\frac{dE^*}{dt} = \frac{- (I_{Kf^*} + I_{Ks^*} + I_h + I_{pump^*} + I_{Lk^*} - I_{BB} - C_{myelin} \times dE/dt)}{C_{ax}} \tag{8.2}
\]

Figure 8.1: Adapted from Howells et al. (2012) Figure 1. The Bostock model of the human motor axon. Schematic description of the key nodal and internodal features of the mathematical model. Voltage-gated channels: sodium (transient (Na) and persistent (Nap)); potassium (fast (Kf) and slow (Ks)); HCN (H). Na+/K+-ATPase pump (Ipump). Ohmic leak conductance (Lk). Capacitance of: axolemma (internodal (Cax), nodal (Cn)); myelin sheath (Cm). Barrett–Barrett conductance (R_{BB}) through and under the myelin sheath.

Membrane Potentials:

\[
\frac{dE}{dt} = \frac{- (I_{Na} + I_{Kf} + I_{Ks} + I_{Lk} + I_{pump} + I_{external} + I_{BB})}{(C_n + C_{myelin})} \tag{8.1}
\]

\[
\frac{dE^*}{dt} = \frac{- (I_{Kf^*} + I_{Ks^*} + I_h + I_{pump^*} + I_{Lk^*} - I_{BB} - C_{myelin} \times dE/dt)}{C_{ax}} \tag{8.2}
\]
Currents:
\[
I_{Na} = \frac{P_{Na}(m^3h + \frac{P_{Na}^2}{RT}m^2)}{RT} \left( Sel_{Na} \left( [Na]_o - [Na]_i e^{\frac{E_{K}}{RT}} \right) + (1 - Sel_{Na}) \left( [K]_o - [K]_i e^{\frac{E_{Na}}{RT}} \right) \right) \left( 1 - e^{\frac{E_{Na}}{RT}} \right)
\]

\[
I_{Kf} = G_{Kf}n^4 (E - E_{Kf}) \\
I_{Kf^*} = G_{Kf} n^4 (E^* - E_{Kf}) \\
I_{BB} = G_{BB} (E - E^*) \\
I_{Ks} = G_{Ks} s^4 (E - E_{Ks}) \\
I_{Ks^*} = G_{Ks} s^4 (E^* - E_{Ks}) \\
I_h = G_h q (E^* - E_h)
\]

\[
I_{Lk} = G_{Lk} (E - E_r) \\
I_{Lk^*} = G_{Lk^*} (E^* - E_r)
\]

Reversal Potentials:
\[
E_x = \frac{\ln \left( \frac{[K]_i + Sel_x [Na]_o - Sel_x [K]_o}{[K]_i + Sel_x [Na]_i - Sel_x [K]_i} \right)}{F} \times RT, \text{ for } x = K_f, K_s, h
\]

Note equation 8.4 differs from the published version (Howells et al., 2012) in that RT appears in the numerator as should be the case according to the GHK voltage equation.

Channel gating:
\[
\frac{dm}{dt} = \alpha_m (1 - m) - \beta_m m
\]

and similarly for \( m_p, h, n, s, n^*, s^*, q \)

\[
\alpha_m, \alpha_m, \alpha_n, \alpha_s = \frac{A(E - B)}{1 - e^{(B - E)/C}} \quad (8.5)
\]

\[
\alpha_h, \beta_m, \beta_m, \beta_n, \beta_s = \frac{A(B - E)}{1 - e^{(E - B)/C}} \quad (8.6)
\]

\[
\beta_h = A/1 + e^{(B - E)/C}
\]

\[
\alpha_q = A e^{(E - B)/C} \quad (8.8)
\]

\[
\beta_q = A/ e^{(E - B)/C} \quad (8.9)
\]

Rate Constant Parameter Values:

Other Parameter Values:
Table 8.1: Voltage and time dependent parameters for the rate constants, $\alpha$ and $\beta$ for motor and sensory (bracketed values) axons. Adapted from Table 2 of Howells et al. (2012).

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<tr>
<th></th>
<th>A(ms$^{-1}$, at 36 °C)</th>
<th>$Q_{10}$</th>
<th>B(mV)</th>
<th>C(mV)</th>
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<tr>
<td>$\alpha_m$</td>
<td>6.54(6.25)</td>
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<td>10.3</td>
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<td>$\beta_m$</td>
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<td></td>
<td>-22.8(-22.6)</td>
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<td>$\alpha_{mp}$</td>
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</tr>
<tr>
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<td>2.9</td>
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<td>$\beta_n$</td>
<td>0.0393</td>
<td></td>
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<td>7.35</td>
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<td>$\alpha_s$</td>
<td>0.00563</td>
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<td>$P_{NaN}$ ($cm^3s^{-1} \times 10^{-9}$)</td>
<td>4.35</td>
<td>4.35</td>
<td></td>
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</tr>
<tr>
<td>$P_{Na^+_g}$ (%)</td>
<td>1.07</td>
<td>1.07</td>
<td></td>
<td></td>
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<tr>
<td>$G_{KS_N}$ (nS)</td>
<td>56.7</td>
<td>29.1</td>
<td></td>
<td></td>
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<tr>
<td>$G_{KS_I}$ (nS)</td>
<td>0.57</td>
<td>1.74</td>
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</tr>
<tr>
<td>$G_{KIN}$ (nS)</td>
<td>18.2</td>
<td>19.4</td>
<td></td>
<td></td>
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<tr>
<td>$G_{KII}$ (nS)</td>
<td>207</td>
<td>205</td>
<td></td>
<td></td>
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<tr>
<td>$G_H$ (nS)</td>
<td>2.95</td>
<td>4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$G_{LKN}$ (nS)</td>
<td>1.97</td>
<td>1.69</td>
<td></td>
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</tr>
<tr>
<td>$G_{LKI}$ (nS)</td>
<td>4</td>
<td>3.65</td>
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<td></td>
</tr>
<tr>
<td>$G_{BB}$ (nS)</td>
<td>35.9</td>
<td>40.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Resting voltages</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_{IR}$ (mV)</td>
<td>-84.6</td>
<td>-81.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_{NR}$ (mV)</td>
<td>-84.4</td>
<td>-80.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pump currents</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$i_{Pump}$ (nA)</td>
<td>$-7.86 \times 10^{-3}$</td>
<td>$-4.3 \times 10^{-3}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$n_{Pump}$ (nA)</td>
<td>$-3.33 \times 10^{-2}$</td>
<td>$-5.44 \times 10^{-2}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Channel Selectivities</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Sel_{Na}$</td>
<td>0.9</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Sel_{Kf}, Sel_{Ks}$</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Sel_h$</td>
<td>0.097</td>
<td>0.097</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ion concentrations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$[Na^+]_i$ (mM)</td>
<td>9</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$[Na^+]_o$ (mM)</td>
<td>144.2</td>
<td>144.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$[K^+]_i$ (mM)</td>
<td>155</td>
<td>155</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$[K^+]_o$ (mM)</td>
<td>4.5</td>
<td>4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Capacitances</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_n$ (pF)</td>
<td>1.4</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{myelin}$ (pF)</td>
<td>1.55</td>
<td>1.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{ax}$ (pF)</td>
<td>327</td>
<td>327</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 8.2**: Constant parameters for the Bostock model of myelinated nerve. Adapted from Table 1 Howells et al. (2012).
8.2.2 Adaptation of the Bostock Model to Include Sodium Channel Blockers

Sodium channel particles

\[ \begin{align*}
R & \xrightarrow{\alpha} I \\
\kappa_a & \xrightarrow{\beta} \kappa_a A \\
\beta Y & \xrightarrow{\beta} BI \\
\end{align*} \]

(a) 6 variables to modify drug effect  \hspace{1cm} (b) 3 variables to modify drug effect

Figure 8.2: Channel model including drug bound state. Rates are modified to simulate drug effect. \( \kappa_a \) and \( \kappa_d \) indicate association and dissociation constants of the drug. \( \alpha \) and \( \beta \) are modified as shown with either 4 variables as shown in subfigure (a) and 1 variable as shown in subfigure (b).

In order to simulate the effect of a drug which blocks sodium channels in a state dependent manner, the equations governing the particles \( m \) and \( h \) as described by Hodgkin and Huxley (1952d) and utilised in the model of myelinated nerve described by Howells et al. (2012), must be adapted to account for a bound conformation which is non-conducting and has preferential action over one particle over the other in order to express state dependence. Karoly et al. (2010) have described patch clamp data with a variety of sodium channel blocking compounds and have fitted their data with an altered Hodgkin and Huxley type model taking into account drug bound sodium channel states with variable preference for fast and slow inactivated particles, this modified model is shown in fig 8.2 adapted from Karoly et al. (2010). In fig 8.2a there must be microscopic reversibility as it is a circular model. The variables \( A, D, Y \) and \( X \), are dependent in that when three are chosen the fourth value must be fixed to maintain microscopic reversibility. Hence:

\[ \alpha \times \kappa_a \times A \times \beta \times Y \times K_d = \kappa_a \times \alpha \times X \times \kappa_d \times D \times \beta \]

\[ \Rightarrow A \times Y = X \times D \]

\[ \Rightarrow \frac{A}{D} = \frac{X}{Y} \]
The method used by Karoly et al. (2010) for breaking four dependent parameters into three independent parameters is as follows:

\[ A = C \times Z \]

\[ D = \frac{Z}{C} \]

\[ X = C \times Q \]

\[ Y = \frac{Q}{C} \]

\[ \Rightarrow C^2 = \frac{A}{D} = \frac{X}{Y} \] where \( C \) determines the drug binding rate of affinity between \( O \) and \( I \) states, as well as the rate of gating equilibria between unbound and bound channels, this means that it gives the free energy level of BI state.

\[ \Rightarrow Z^2 = A \times D \text{ and } Q^2 = X \times Y \] where \( Z \) and \( Q \) will modify the height of the energy barrier in the I to/from IB and OB to/from IB transitions, respectively. (If R state is defined as zero level, the level of I is determined by the \( \beta/\alpha \) ratio, and the level of BO is determined by the \( kd/kd \) ratio).

Karoly et al. (2010) in order to apply drug effect to the sodium channel defined a single \( K_a \) and \( K_d \) for all three particles \( m, h \) and \( s \). The \( s \) particle is added by Karoly et al. (2010) to allow description of modification of a slow inactivating sodium channel particle. Karoly et al. (2010) set \( Z=1 \) and \( Q=1 \) for simplicity in their analysis and varied \( C \) independently for each particle CA for “Closed Activation gate stabilizing factor” (applying to the \( m \) particle), CF for “Closed Fast inactivation gate stabilizing factor” (applying to the \( h \) particle) and CS for “Closed Slow inactivation gate stabilizing factor” (applying to their newly introduced \( s \) particle). Thus by varying the values of CA,CF and CS the affinity of the drug and propensity to enter the bound closed state for different states maybe varied. Application of the above principles to the sodium channel particles can be represented as displayed in figure 8.3.

and the dParticle/dt equations must be modified thus (note only the \( O \) states are relevant for calculation of the Na\(^+\) current however the other states must be tracked in as the \( O \) state is dependent upon these):

\[
\frac{dmO}{dt} = (\alpha_m (1 - (mO + mOB + mCB))) - (\beta_m \times mO) - (k_a \times C_{drug} \times mO) + (k_d \times mOB);
\]

\[
\frac{dmOB}{dt} = ((\alpha_m/CA) \times mCB) - (\beta_m \times CA \times mOB) + (k_a \times C_{drug} \times mO) - (k_d \times mOB);
\]

\[
\frac{dmCB}{dt} = \frac{(\beta_m \times CA \times mOB) - (\alpha_m/CA) \times mCB) + ((\alpha_m/CA) \times mCB)}{(k_a \times CA \times C_{drug} \times (1 - (mO + mOB + mCB))) - (k_d/CA \times mCB)};
\]

155
**Figure 8.3:** Na⁺ channel model particles. Adaptation of the Hodgkin and Huxley a) m particle and b) h particle to incorporate a drug bound state. c) an s particle may be optionally added to the equations to represent a slow inactivating particle. C - closed/non-conducting state, O - open/conducting state, CB-closed and bound to drug state, OB- open and bound to drug state. As described by Karoly et al. (2010), CA CF CS represent the gate stabilising factors, Ka and Kd represent association and dissociation rate constants of the drug. The $\alpha$ and $\beta$ for each particle represent the voltage dependent forward and backward rates between states.

\[
\frac{dmO}{dt} = (\alpha_m (1 - (hO + hOB + hCB))) - (\beta_m \times hO) - (k_a \times C_{drug} \times hO) + (k_d \times hOB);
\]

\[
\frac{dmOB}{dt} = ((\alpha_m/CA) \times hCB) - (\beta_m \times CA \times hO) - (k_a \times hCB);
\]

\[
\frac{dhO}{dt} = (\alpha_h (1 - (hO + hOB + hCB))) - (\beta_h \times hO) - (k_a \times C_{drug} \times hO) + (k_d \times hOB);
\]

\[
\frac{dhOB}{dt} = ((\alpha_h/CF) \times hCB) - (\beta_h \times CF \times hO) - (k_a \times CF \times C_{drug} \times hO) - (k_d \times hOB);
\]

\[
\frac{dsO}{dt} = (\alpha_s (1 - (sO + sOB + sCB))) - (\beta_s \times sO) - (k_a \times C_{drug} \times sO) + (k_d \times sOB);
\]

\[
\frac{dsOB}{dt} = ((\alpha_s/CS) \times sCB) - (\beta_s \times CS \times sOB) - (k_a \times CS \times C_{drug} \times sO) - (k_d \times sOB);
\]

\[
\frac{dsCB}{dt} = ((\alpha_s/CS) \times sCB) - (\beta_s \times CS \times sOB) - (\alpha_s/CS \times sCB) + (k_a \times CS \times C_{drug} \times (1 - (sO + sOB + sCB))) - (k_d/CS \times sCB);
\]

The slow inactivation gate is omitted from the revised Bostock model detailed here due to practical limitations of computing power and the postulated action of the compounds of interest on the fast inactivated state. A slow inactivation particle can however optionally be added with appropriate parameters.

With the above modified dParticle/dt equations, the sodium current equation ($I_{Na}$ in equations 8.3) must be modified with the value of $m$ and $h$ being replaced by the new values $mO$ and $hO$ respectively, note in this equation the persistent sodium conductance is not changed:
\[ I_{Na} = P_{Na} \left( mO^3hO + \frac{P_{Na}E_m}{100} \right) \frac{K_{Na}^2}{[Na]_o} \left( [Na]_o - [Na]_i \right) \frac{e^{\frac{E_{fr}}{RT}}}{\left( 1 - e^{\frac{-E_{fr}}{RT}} \right)} \]

The new parameters required for the model are:

<table>
<thead>
<tr>
<th>Description</th>
<th>Parameter</th>
<th>Value range</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug association rate constant:</td>
<td>( K_a ) (s(^{-1})µM(^{-1}))</td>
<td>0.005:0.5</td>
<td>Concentration dependent</td>
</tr>
<tr>
<td>Drug dissociation rate constant:</td>
<td>( K_d ) (s(^{-1}))</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>Concentration of drug:</td>
<td>( C_{drug} ) (µM)</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>Closed state stabilising factors:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activation gate:</td>
<td>CA</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Fast inactivation gate:</td>
<td>CF</td>
<td>1:50</td>
<td>Optional (depending on model)</td>
</tr>
<tr>
<td>Slow inactivation gate:</td>
<td>CS</td>
<td>1:50</td>
<td>Optional (depending on model)</td>
</tr>
</tbody>
</table>

Table 8.3: Drug adapted model parameters for sodium channel inhibition
8.2.3 Implementation of the mathematical model

The equations above were developed and tested in the statistical programming language R (Team, 2012) and ordinary differential equations solved using the R package deSolve. Model parameters used were are those modelled in human motor and sensory nerve from Howells et al. (2012), and drug parameters for classic fast inactivated state selective sodium channel blockers are taken from Karoly et al. (2010).

Once a working model with sodium channel modulation was developed, the equations and parameters described in this section were kindly implemented in the program QTRACp and the memfit module by Prof Hugh Bostock. This implementation has the advantage of running in a tried and tested environment, and offered huge improvements in speed of modelling due to the compiled nature of QTRAC rather than the interpreted programming language of R.

8.3 Results

8.3.1 Parameters used to simulate drug effect

In order to compare the different effects that drugs active on the sodium channel may have on the nerve excitability profile, parameters of the new model are varied in order to represent different types of sodium channel blockade. This modelling is then compared with the results of the human experiments described in chapter 4. The range of sodium channel blocker parameters used in this model are as shown in table 8.4. Carbamazepine is modelled as a fast inactivated state blocker with fast kinetics according to Karoly et al. (2010) with DKa set to 0.5, DKd set to 100, CA set to 1, and CF set to 10. Most classic anticonvulsants of which Carbamazepine is one are considered to be in this class. A sodium channel blocker with theoretical preference for the resting state is also modelled with CA set to 10 and CF set to 1. A non-state selective blocker (as represented by Tetrodotoxin (Kiernan et al., 2005a)) is modelled according to the unaltered Bostock model by adjusting the sodium conductance to 80% of its normal value.

In the QTRAC model DKa and drug concentration are combined to simplify implementation and as this has no effect on the equations as DKa is the only drug concentration dependent parameter.
Table 8.4: Model parameters simulating state-selective sodium channel inhibition

8.3.2 Modelling of motor nerve with sodium channel inhibition

Figure 8.4 shows the results of modelling on the nerve excitability profile. The first column represents the human drug trial results, the second column represents a fast inactivated state blocker such as carbamazepine, the third column represents a resting state blocker without preference for the fast inactivated state, and the fourth column represents an unselective blocker such as tetrodotoxin. It should be noted that the parameters of the basic Bostock model are of those most recently published rather than fitted to trial data in chapter 4.

The charge-duration properties and hyperpolarising threshold electrotonus differentiate between state selective sodium channel blockade in a model of myelinated motor nerve.

Differences between the modelled nerve excitability profile between fast inactivated state block (Flb), resting state block (Rsb) and un-selective channel block (Usb) can be seen in the charge-duration plot and hyperpolarising threshold electrotonus.

The charge-duration plot shows a change in SDTC and Rheobase for both Rsb and Usb models but only a small change in Rheobase for the Flb with very little effect on SDTC. The closest model to the human motor data can be seen to be Flb. Human motor data show no significant change in the Rheobase or SDTC (see figure 4.7), although the mean results as seen in figure 8.4 do show differences albeit not significant.

Hyperpolarising threshold electrotonus shows an increased amplitude in the models of Rsb and Usb, however in the model of Flb there is a decrease in the amplitude. Again although there is no significant effect on hyperpolarising threshold electrotonus in the human means show a minor decrease in amplitude, the closest match being again with the Flb model.
Depolarising electrotonus, recovery cycle and IV curve show changes characteristic of sodium channel blocking but do not differentiate between state preference

Other measures of excitability shown in figure 8.4 demonstrate essentially similar effects between models and the human motor data. Differences between the models and the human data are seen in the subexcitability which does not change significantly in the human but shows decreased amplitude in all models.

Parameter optimisation

The memfit module of QTRACp can be used to optimise parameters within the model using an iterative least root mean square error method. This technique was first applied to the pre-drug control motor data in humans and the model parameters providing a best fit for the control data are shown in table 8.5. It should be noted that the relatively large change in the internodal fast potassium conductance is not unexpected and likely results from two factors, firstly that there isn’t any one measure of excitability specifically sensitive to this parameter and secondly that the model is only two compartment and in reality a third compartment representing the juxtaparanode is needed to adequately deal with aspects related to this potassium conductance.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Original</th>
<th>Optimised</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{Na%}$ (%)</td>
<td>1.07</td>
<td>0.655</td>
</tr>
<tr>
<td>$G_{Kf}$ (nS)</td>
<td>207</td>
<td>62.5</td>
</tr>
<tr>
<td>$G_{Kn}$ (nS)</td>
<td>1.97</td>
<td>3.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Resting voltages</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIR (mV)</td>
</tr>
<tr>
<td>ENR (mV)</td>
</tr>
</tbody>
</table>

Table 8.5: Constant parameters for the Bostock model of myelinated motor nerve optimised for pre-drug results presented in chapter 4.

Building upon this, optimisation was then performed allowing variation in the drug parameters to fit the motor results at 4 hours after carbamazpine. Optimisation produced a modest 20% improvement in fit with parameters of DKA 0.6, DKd 100, CA 1.2 and CF 10, this is shown in table 8.6. These parameters are extremely close to those described by Karoly et al. (2010) for a fast-inactivating state selective blocker, and as such provide support for the approach used.
The main problems with this approach discussed in section 8.4, and are essentially that the model does not have sufficient differences in the traces to appropriately use a least root mean square approach despite the high level significance of the findings. For this reason, a graphical approach to elucidate the main features proves more helpful.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimised model parameter</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Association rate constant (DKa)</td>
<td>0.6</td>
<td>$s^{-1}\mu M^{-1}$</td>
</tr>
<tr>
<td>Dissociation rate constant (DKd)</td>
<td>100</td>
<td>$s^{-1}$</td>
</tr>
<tr>
<td>Resting channel preference (CA)</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Fast inactivated preference (CF)</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Table 8.6: Model parameters showing best fit to human trial data post carbamazepine.

Modelling of fast inactivated state sodium channel blockade differentiates the effect from resting state block and un-selective channel block in motor nerve and most closely matches the effects of Carbamazepine in human motor nerve

It should be noted that the human trial has limited power due to the number of individuals involved, and while being sensitive to parameters with major changes such as the depolarising threshold electrotonus, parameters such as Rheobase which may show a subtle change in means may not reach statistical significance. For this reason the results in figure 8.4 are shown as means at peak drug effect and the similarity of the pattern of changes matched to the drug model.

The pattern of changes seen in the depolarising threshold electrotonus and IV curve are very similar between all models and the human data, the superexcitability likewise shows similar changes in all models and the human data whereas the sub-excitability while similar between models does not show a similar change in the human data. Of most interest are the patterns of changes seen in the charge-duration plot and the hyperpolarising threshold electrotonus, where the fast inactivated blockade model matches the human data, whereas the un-selective and resting state blockade models show differences. Overall this approach convincingly demonstrates, that modelling of fast inactivated state blockade, most closely resembles the changes seen in the human motor nerve as a result of Carbamazepine. The only remaining discrepancy being in the effects on sub-excitability.
Figure 8.4: Effect of sodium channel inhibition of the nerve excitability profiles of motor axons. Axes are labelled, including units, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000). Column a) Human experimental results before (black line) and 4h after administration of carbamazepine (red line). Columns b) - d) in the figure compare nerve excitability parameters of the standard mathematical model of motor nerve (black line) compared with the modelled effect of different modes of sodium channel inhibition (red line). The model is a simplified description of nerve and describes only a single type of sodium channel. The drug adapted model introduces 4 new variables to the standard model (most recently updated by Howells et al. (2012)). The variables are drug association rate constant (DKa), dissociation rate constant (DKd), resting channel preference (CA) and fast inactivated preference (CF) as described by Karoly et al. (2010). Column b) shows drug adapted model parameters for a fast inactivated state blocker (DKa 0.5, DKd 100, CA 1, CF 10). Column c) shows drug adapted model parameters for a resting state blocker (DKa 0.5, DKd 100, CA 10, CF 1). Column d) Models an un-selective sodium blocker using the standard model by reducing nodal sodium permeability to $3.48 \times 10^{-9}$. 

\[ 3.48 \times 10^{-9} \]
8.3.3 Modelling of sensory nerve with sodium channel inhibition

Figure 8.5 shows the results of modelling on the sensory nerve excitability profile, as presented for the motor nerve.

Modelling of sodium channel blockade in sensory nerve does not distinguish well between state-selectivity

Figure 8.5 shows that the patterns of change in the nerve excitability profile as a result of selective blockade of F1b, RSb and USb show little difference. There is a difference in the magnitude of the effect seen in the hyperpolarising threshold electrotonus, however the effect is in the same direction and by itself this difference is of limited use in differentiating types of state selectivity.

Irrespective of state-selectivity, sodium channel blockade in the sensory nerve model causes; reductions in both depolarising and hyperpolarising threshold electrotonus; increase in rheobase and reduction in SDTC; a reduction in super-excitability and to a lesser extent sub-excitability; an increase in IV slope in both the depolarising and to a lesser extent hyperpolarising sections.

Modelling partially explains the human sensory nerve carbamazepine effect

Results of the modelling of drug effect show major similarities between the human sensory data and the drug models as seen in figure 8.5. The major difference between the modelled data and the human data is in the initial phase of depolarising threshold electrotonus, where the human data show no change in TEd peak whereas the models show a reduction in TEd peak. This feature therefore remains unaccounted for by the current drug model.

The conclusion from the modelling of sensory nerve drug effect and comparison with the human data therefore is that while the model may indicate a sodium channel blockade as a likely explanation for the human data, distinction between state-selectivity is not well achieved and there remain features unexplained by the drug adapted model. It is for this reason parameter optimisation has not been attempted to explain the sensory data, addition of a slow inactivated state to the model may prove fruitful in this regard and is discussed in section 8.4.
Figure 8.5: Effect of sodium channel inhibition of the nerve excitability profiles of sensory axons. Axes are labelled, including units, according to accepted nerve excitability convention of the TROND protocol as described by Kiernan et al. (2000). Column a) Human experimental results before (black line) and 4h after administration of carbamazepine (green line). Columns b) - d) in the figure compare nerve excitability parameters of the standard mathematical model of sensory nerve (black line) compared with the modelled effect of different modes of sodium channel inhibition (green line). The model is a simplified description of nerve and describes only a single type of sodium channel. The drug adapted model introduces 4 new variables to the standard model (most recently updated by Howells et al. (2012)). The variables are drug association rate constant (DKa), dissociation rate constant (DKd), resting channel preference (CA) and fast inactivated preference (CF) as described by Karoly et al. (2010). Column b) shows drug adapted model parameters for a fast inactivated state blocker (DKa 0.5, DKd 100, CA 1, CF 10). Column c) shows drug adapted model parameters for a resting state blocker (DKa 0.5, DKd 100, CA 10, CF 1). Column d) Models an un-selective sodium blocker using the standard model by reducing nodal sodium permeability to $3.48 \times 10^{-9}$ cm$^3$s$^{-1}$. 
8.4 Discussion

8.4.1 Mathematical modelling of sodium channel inhibition

The results presented here demonstrate that the mathematical model proposed by Karoly et al. (2010) to simulate sodium channel inhibition by drugs, when incorporated into the Bostock model of myelinated nerve, achieves partial success in modelling the biological effects of carbamazepine. This study is the first time which mathematical modelling has been used in order to try and explain the mechanistic action of a therapeutic drug on the electrophysiology of peripheral nerve in humans.

The findings indicate that the effect of carbamazepine effect in human motor nerve is most closely matched by preferential binding to the fast inactivated state of the modelled sodium channel. In sensory nerve modelling, inhibition of sodium channels also matches the results seen, however this match is poorer with certain features showing a mismatch as is notable for example in the early phase of depolarising threshold electrotonus. In contrast to motor nerve, sensory nerve modelling with the TROND protocol does not appear to clearly distinguish features of state-selectivity with the results of modelling an un-selective state blocker very similar to those of modelling a fast-inactivated state blocker or a resting state blocker (Figure 8.5).

8.4.2 Mathematical modelling limitations

Studies reported so far which have used mathematical modelling of nerve excitability studies are outlined in Table 8.7.

The studies shown in table 8.7 have successfully applied motor nerve modelling. By contrast, success with sensory nerve modelling has been more elusive. Despite the proposal of a model for human sensory nerve there have been no studies using this model to explain perturbations of nerve excitability as a result of drug or pathology. This is in part due to only relatively recent proposition of a human sensory model and in part likely due to more substantial differences between motor and sensory nerve. Differences in response of motor and sensory nerve to an external drug are clearly seen for example in Kiernan et al. (2005a) but to date are not explained (however the similarity between the unselective state blocker sensory modelling shown in figure 8.5 and the sensory nerve findings after tetrodotoxin are notable).

For mathematical modelling to be successful, firstly a model requires a biologically plausible mechanism (parameters) within to be able to describe the changes, secondly data with sufficient differences to be able to assess the effects of varying these parameters on the fit to experi-
<table>
<thead>
<tr>
<th>Name</th>
<th>Year</th>
<th>Context</th>
<th>Modelling Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kiernan et al. (2005a)</td>
<td>2005</td>
<td>Pufferfish poisoning</td>
<td>Reduced Na⁺ conductances</td>
</tr>
<tr>
<td>Kanai et al. (2006)</td>
<td>2006</td>
<td>Amyotrophic Lateral Sclerosis</td>
<td>Reduced K⁺ conductances</td>
</tr>
<tr>
<td>Lin et al. (2008)</td>
<td>2008</td>
<td>Acute intermittent porphyria</td>
<td>Reduced Ih</td>
</tr>
<tr>
<td>Boland et al. (2009)</td>
<td>2009</td>
<td>Spinal cord injury</td>
<td>Increased nodal leak conductance</td>
</tr>
<tr>
<td>Farrar et al. (2011)</td>
<td>2011</td>
<td>Spinal muscular atrophy</td>
<td>Reduced internodal length</td>
</tr>
<tr>
<td>Tomlinson et al. (2012)</td>
<td>2012</td>
<td>Benign familial neonatal epilepsy</td>
<td>Reduction of slow K⁺ conductance</td>
</tr>
<tr>
<td>Boërio et al. (2014)</td>
<td>2014</td>
<td>Normal controls</td>
<td>Increased fast K⁺ conductance</td>
</tr>
<tr>
<td>Tomlinson et al. (2016)</td>
<td>2016</td>
<td>Episodic ataxia type II</td>
<td>Increased Barrett-Barrett conductance</td>
</tr>
</tbody>
</table>

Table 8.7: A table showing studies in which mathematical modelling of motor nerve has contributed to the conclusions. It should be noted that modelling provides suggestive explanations which require collaborative evidence.

mental data. If, with these considerations, modelling is unsuccessful the conclusion must be that either the mechanism is inaccurate, there is an element of the model which is missing or wrong, or that the assumptions underlying the model are in some way invalid.

On the first point the addition of a plausible mechanism the aim has been achieved by adaptation of the model and has demonstrated some success albeit suboptimal.

On the second point although the findings of chapter 4 are highly significant and are able to separate individual subjects before and after carbamazepine, in terms of the differences to the model the changes are not large. When compared to other scenarios in which the Bostock model has been applied to explain real world results such as in the case of individuals poisoned by tetrodotoxin (Kiernan et al., 2005a) or in individuals with renal failure pre- and post-dialysis (Krishnan et al., 2005) differences enabling successful explanation of results have been of larger amplitudes allowing the model more room to assess for effects of parameter optimisation. In both these situations nerve dysfunction has been unavoidable as a result of pathology; in the situation of a trial in healthy human individuals as reported here, safety is the number one priority, hence the dosages of carbamazepine achieved are within the therapeutic range rather than the toxic range. It is likely that had higher serum levels of Carbamazepine been achieved larger effects on the nerve excitability profile would have been seen.

This limitation on the size of the effects and only modest success with the drug adapted model, makes conclusions from modelling somewhat tentative. Results have been presented in a way that makes visual comparison of the main features of the nerve excitability profile comparable between models and experimental data.
Modelling has not been applied to experimental data from the ex-vivo rat model for two reasons; firstly that there is no existing model of this system with validated parameters; and secondly the sensory model in human provides minimal distinction between state-selectivity, and this is therefore likely within the rat model.

Addition of slow inactivation of the sodium channel is also described in the equations presented, but has not been implemented here due to the need to adapt the underlying Bostock model to a higher degree. Introduction of this inactivation and drug effect to it may help provide better understanding of the effects seen here, and may for example provide an explanation for the discrepancy seen in the early phase of depolarising electrotonus in the sensory model of drug effect.

On the third point there is an intrinsic assumption of nerve excitability studies as performed here that the nerve fibres are a homogenous group with essentially identical properties. While the success of the approach with motor nerve argues that this assumption is valid, one reason for a lack of success for the sensory nerve is that there may be much more heterogeneity between fibres. This is exemplified by the ‘notch’ which may be seen in the early depolarising threshold electrotonus which results from excitation of some sensory fibres by the conditioning pulse. Another possible explanation is the effects of FHF2B on the NaV1.6 channel (Wittmack et al., 2004), whether this also in itself modifies the action of sodium channel blockers is unknown.

8.4.3 Approaches to confirm the findings presented

In order to overcome the small absolute effect size a possible approach would to that of performing nerve excitability studies in-vivo in the mouse model (Boërio et al., 2011), which would allow higher serum concentrations of carbamazepine to be achieved in a validated model outside of the human and a physiologically well controlled environment. An alternative approach is to study those patients who achieve high serum concentrations of carbamazepine either through a clinical intent or through overdose.

This study was designed to exclude possible underlying abnormalities in nerve and measure the pure effects of carbamazepine in healthy subjects. Now that this has been achieved testing of patients treated both acutely and chronically carbamazepine would provide further insight into the clinical role of the drug, and allow assessment of the nerve in the chronically treated state.

Alternative protocols of nerve excitability have been used recently to increase the power of threshold tracking to detect drug effect. The approach of using ramp currents to increase the inactivated state of sodium channels has demonstrated the state selectivity of Lidocaine and surprisingly Tetrodotoxin in a rodent ex-vivo preparation (Vastani et al.,
2013). This technique is also susceptible to mathematical analysis as described here and application of this may provide further insight into state-selectivity, as well as being a technique applicable to human trials.

Addition of the slow inactivated state to the Bostock model and allowing drug to bind to this state is also desirable as a future goal, especially as the anticonvulsant lacosamide is thought to work via this mechanism (Errington et al., 2008).

8.4.4 Potential applications of mathematical modelling

Pharmacogenetics and nerve excitability

Pharmacogenetic variations in individuals response to a medication maybe in part at least determined by genetic differences (Szoeké et al., 2006). For example Carbamazepine resistance has been associated with a polymorphism in SCN1A (Tate et al., 2005). While SCN1A is not thought to be expressed in peripheral nerve, peripheral signatures of a central pathology as in GEFS+ due to an SCN1B mutation have been detected in peripheral nerve using the nerve excitability profile (Kiernan et al., 2005b). There is also evidence recent that NaV1.6 may be implicated in epileptogenesis in a model of induced seizures (Hargus et al., 2013).

Carbamazepine is also used in the context of pain, of more relevance to the peripheral nerve. Although pain is primarily thought to be mediated by un-myelinated C and thinly myelinated Aβ peripheral nerve fibres which express a different repertoire of sodium channels, they do however share some and recent evidence indicates the possibility of greater overlap than previously thought (Ramachandra et al., 2013).

Considering these points nerve excitability and mathematical modelling may have a place and prove fruitful in assessing individuals response to medication aimed at sodium channel inhibition, and correlation with pharmacogenetics would be interesting.

Nerve excitability and modelling as a translational biomarker for drug trials

At the cost of around a billion dollars to develop a drug, with the majority spent in clinical phases (Adams and Brantner, 2006), the drugs industry is constantly seeking ways to reduce cost. As discussed in section 8.1.2, a limitation of current drug development is the lack of translatable biomarkers. Chizh et al. (2009) highlight that only around 10% of candidate molecules progressing into human trials finally achieve regulatory approval, the authors highlight translatable biomarkers directly related to mechanism of action and also improvement in pharmacokinetic/pharmacodynamic assessments as key tools to achieve early decisions in the early human stages of drug development. The costs have
become so large indeed that, even for common conditions such as epilepsy (with 30% of seizures resistant to pharmacotherapy) the development of new anticonvulsants is at risk of being uneconomical. Pain has been turned to as an alternative development pathway for many candidate molecules previously considered for epilepsy (Waszkielewicz et al., 2011).

The data presented here provide evidence that the nerve excitability profile fulfils just that niche as being a translatable biomarker providing insight into mechanism of action and providing PK/PD information. This study provides proof of principle for this in the context of sodium channel inhibiting compounds, however there is no reason that the approach used here cannot be applied to other drugs active on peripheral nerve ion channels for example potassium channels.
Chapter 9

Conclusion

The results presented here provide evidence to support the four main hypotheses. The effects of carbamazepine can be reliably detected by the nerve excitability profile in the peripheral nerve of healthy volunteers. The hypothesised increase in S2 accommodation and SDTC, with a decrease in rheobase is seen in human sensory nerve, however a slightly different pattern of changes are seen in motor nerve. The effects of the metabolite carbamazepine-epoxide are demonstrable in ex-vivo rat sensory nerve, but at the relevant concentrations to those seen in human blood, do not show significant effect in this study, supporting the conclusion that it is carbamazepine rather than its metabolite responsible for the changes seen in the human trial.

Adaptation of the nerve excitability studies to allow for assessment of frequency dependence, shows a recordable increase in threshold related to frequency in human and rat. The drugs lidocaine, carbamazepine and tetrodotoxin show differing effects in the ex-vivo rat sensory nerve with these frequency-dependent protocols. These results provide promise for assessing frequency-dependent properties of drugs in humans with this technique.

In all the protocols applied to human nerve, in which changes have been seen as a result of carbamazepine, the direction of effect has been to decrease excitability or, to put in another way, to result in the requirement for a stronger depolarisation in order to generate the same response from the nerve. The largest amplitude, consistent response to carbamazepine for both human motor and sensory nerve as well as in the rat ex-vivo nerve is the reduction in excitability to periods of more prolonged sub-threshold depolarisation (e.g. 100-200ms).

The findings provide evidence, for a differential excitability effect of carbamazepine on human motor and sensory nerve at their resting physiological state. The explanation favoured by the author, is that this is due to differing resting membrane potentials of human sensory and motor nerve. Other mechanisms must however be considered, the effects of Fibroblast growth factor homologous factor 2b (FHF2B), which is se-
lectively expressed in sensory nerve and modulates sodium channels, being one possibility.

Changes in nerve excitability results are often not readily susceptible to analysis through visual inspection alone and require a complex mathematical model to interpret. The results of minor perturbations in parameters in the model often produce changes in the profiles which are not easily or may not be at all intuitable from initial inspection of the curves. The adaptation of the mathematical model presented here attempts to address this as has been shown with some success. It is shown that adding drug binding and modelling preferential inactivated state block, resting state block and unselective block improves the ability to study drug effect and that carbamazepine is indeed most closely modelled as an inactivated state blocker.

It is hoped that this proof of principle, namely that the technique described here are a sensitive translatable biomarker allowing mechanistic insight, will promote the technique as a useful tool in drug development.

The limitation of threshold tracking technique utilised in this thesis is to peripheral large myelinated nerve, however the main target site for many ion channel active medications for example in epilepsy or pain is the central axons or small peripheral axons respectively. This may be thought to render a technique which does not assess these sites less relevant, however there are commonalities between central and large fibre peripheral axon ion channel expression, as well as between peripheral small or un-myelinated and large myelinated axons, both in development and maturity. An example is benign familial neonatal convulsions which is essentially a disorder with clinical manifestations in the central nervous system but with a signature detectable in the peripheral nerve.

Further avenues of investigation would be to study a wider range of ion channel active medications, refine the mathematical model further and develop new current protocols to draw out other aspects relevant to drug action. Current measures to monitor drug therapy in patients, such as measuring drug levels do not provide any insight into actual physiological action on an individual basis these techniques offer; whether the technique may be of useful as a therapeutic marker is unknown but would also be an interesting avenue of investigation.
Chapter 10

Bibliography


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