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Axon excitability testing shows increased I_H activity in populations of slow versus fast motor axons of the rat

by

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To my wifey.

Abstract

Despite extensive knowledge of variations in motoneuron (MN) soma and muscle properties across different healthy muscles or motor units, there is comparatively little knowledge about variations in motor axon electrophysiology across different axon groups. Axon excitability testing (AET) is an *in vivo* method which indirectly examines motor axon electrophysiology. We used AET in Sprague-Dawley rats to compare axons innervating tibialis anterior ("fast" motor axons) to axons innervating soleus ("slow" motor axons). We found that fast and slow motor axons differ significantly in their accommodation to hyperpolarizing currents, and in their post-spike excitability oscillation. Specifically, we found compelling evidence that slow motor axons have greater activity of the hyperpolarization-activated inwardly rectifying cation conductance (I_H) than fast motor axons. Since fast and slow motor axons have different daily activity patterns, this foreshadows the possibility of activity-dependent plasticity in at least one ionic conductance of the motor axon.

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Abbreviations

The following abbreviations have been used in this thesis.

AET.....axon excitability testing

ALS.....amyotrophic lateral sclerosis

CAP.....compound action potential

CMAP.....compound muscle action potential

CV.....conduction velocity

HCN.....hyperpolarization-activated cyclic nucleotide-gated

I_H.....hyperpolarization-activated inwardly rectifying cation conductance

I/V.....current-threshold

MN.....motoneuron

RC.....recovery cycle

RRP.....relative refractory period

SCI.....spinal cord injury

SDTC.....strength-duration time constant

SOL.....soleus

TA.....tibialis anterior

TE.....threshold electrotonus

TEd.....depolarizing threshold electrotonus

TEh.....hyperpolarizing threshold electrotonus

Chapter 1

Introduction and Motivation

1.1 Big Picture

The mammalian neuromuscular system is charged with the task of executing smooth force outputs in a great range of environmental conditions and in a timely manner. This requires impeccable coordination of multiple processes in the central and peripheral nervous system. In the healthy peripheral nervous system, the motor axon offers faithful transmission of electrical signals passing from the parent motoneuron (MN) soma to its innervated muscle fibers. Typically, motor axons have a capacity for sending signals at a rate that is 10x the highest rate useful for their muscle fibers (Erlanger and Gasser 1937, Kernell 2006). The bioelectric properties of motor axons are not immutable, however. This is exemplified by studies in which conduction velocity (CV) has been found to change after altered activity patterns, or simply when CV has been recorded in the

same axon over a period of several months (Swadlow 1982, Carp *et al.* 1994, Debanne *et al.* 2011). In addition, in diseases such as amyotrophic lateral sclerosis (ALS), certain axon bioelectric (electrophysiological) properties are altered which can threaten the faithful signal propagation in motor axons.

A deeper understanding of motor axon bioelectric variations in healthy states may very well improve our understanding of the pathophysiology of motor axons in certain diseases. However, there have historically been large technical difficulties associated with the investigation of motor axon bioelectric properties besides CV, and there are many questions about motor axon properties which need to be systematically addressed. To begin the task of understanding motor axon variations and adaptations, this thesis used a new automated version of a technique called axon excitability testing (AET). AET is an *in vivo* method which uses axon threshold properties to infer the functioning of distinct ionic conductances along the axon proper. In this study, we used AET to examine the bioelectric properties of two healthy motor axon populations in the rat, one which innervated the tibialis anterior (TA) muscle and another which innervated the soleus (SOL) muscle. These two axon groups differ substantially in their normal daily activity patterns (Hennig and Lomo 1985, Gorassini *et al.* 2000).

1.2 Basic Science Perspective

1.2.1 A Bias in Methods

It is likely that a bias in methods has inhibited growth of motor axon knowledge.

The diameters of mammalian motor axons are less than half the size of the diameter of their corresponding MN soma. For example, the average diameter of motor axons innervating medial gastrocnemius in adult cats is less than 15 μm, whereas the average diameter of medial gastrocnemius/SOL MN somas in cats exceeds 50 μm (Kernell 2006). This type of discrepancy can create large technical obstacles when probing the electrophysiological properties of mammalian motor axons through the use of microelectrodes. And since electrophysiology plays a prominent role in neurophysiology research, it also plays a prominent role in *creating* new research questions addressing physiological phenomena of interest. In neurophysiology research, if electrophysiology is used more often on the soma than the axon or dendrites, then some of the future research arising from these studies (computational models, molecular studies, additional electrophysiological studies, etc.) will be more dependent on the physiology of the soma than the dendrites or axons, leading to a deficiency not only in neuronal models but in MN physiology as a whole.

One example of this deficiency is found when considering that only MN somatic bioelectric properties have been used to properly classify Burke's three motor unit types in cat medial gastrocnemius, despite axon CV being nearly as successful in classifying fast (fast fatigable or fast fatigue-resistant) and slow motor units as the somatic electrical properties were. For example, in cat MG 86% of the motor units were successfully classified as fast or slow using axon CV, compared to 91% of the motor units using somatic input resistance (Zengel *et al.* 1985). If more axon bioelectric properties were measured, they may have been as

fortuitous as the somatic properties in classifying the conventional three motor unit types. A result of this kind of oversight is that motor axon bioelectric properties may be mistaken as relatively uninteresting in comparison to MN or motor unit physiology. However, axon action potential amplitude and CV were instrumental in Henneman's work on MN recruitment, as well as in Erlanger and Gasser's work on classifying compound nerve action potentials (Erlanger and Gasser 1937, Henneman *et al.* 1965). This underscores the importance of motor axon properties in the history of neurophysiology research.

It is plausible that if a variety of motor axon bioelectric properties could be aptly and efficiently recorded in individual motor axons, and their relationship to other MN or motor unit properties was determined in a variety of muscle nerves and species, then this could generate novel ideas in contemporary neurophysiology research. Due to the many technical limitations in determining the bioelectric properties of individual axons *in vivo*, it is understandable there is little interest in systematically investigating axon properties in the same way that somatic properties of individual neurons, such as afterhyperpolarization (AHP) amplitude, input resistance, rheobase, and membrane time constant, which have been systematically investigated to the point of becoming standard measures in mammalian MN electrophysiology. Nevertheless, the possibility that motor axon properties may co-vary with other MN or motor unit properties remains an intriguing idea. For example, if axon bioelectric properties correlated with one another across entire MN pools, but did not correlate with one another within a particular motor unit type, this would promote the "heuristic appeal" of motor

units being classified into functionally-distinct groups (Zengel *et al.* 1985, Burke 1999). On the other extreme, motor axon bioelectric properties besides CV may remain relatively constant across MN pools, in contrast to the large range found for somatic and muscle unit properties.

1.2.2 Variations in Motor Axons are Under-acknowledged

The bioelectric properties of healthy motor axons are known to vary in some studies (Burke *et al.* 2001, Bae *et al.* 2009, Trevillion *et al.* 2010, Nodera and Rutkove 2012, etc.; see table 2.1) but these variations are considered subtle and not as functionally significant as variations in MN somatic bioelectric properties. Hence, many axon models to date have been largely unconcerned with motor unit type (i.e. Bostock *et al.* 1991, McIntyre 2002). But there are questions that should be raised in regard to the assumed disconnectedness between neuronal compartments – why are "actively coordinated" somatic bioelectric properties fervently studied (i.e. Gustaffson and Pinter 1984, Zengel *et al.* 1985, Gardiner 1993, Munson *et al.* 1997, Kernell 2006, etc.) while variations in axon bioelectric properties often are implicitly assumed to be unimportant or uncoordinated? Functional variations in axons of the central nervous system are now overtly recognized, including activity-dependent plasticity of some axons (Bucher and Goaillard 2011, Debanne *et al.* 2011). Furthermore, there are a number of MN somatic properties which are known to adapt to various chronic activity conditions (Gardiner 2006, Munson *et al.* 1997, etc.). These observations beg the question, *if motor axons learn activities, which motor axon properties can learn and in what ways do they learn*? In contrast with their central nervous system

counterparts, why are peripheral motor axon bioelectric properties assumed to be relatively - for lack of a better word - static? Before questions about motor axon adaptations are explicitly addressed using chronic alterations to activity (see section 2.3), it seems pertinent to first investigate how axon bioelectric properties differ between motor axons with different usage (daily activity) patterns. This was the primary aim of our study.

Current neurophysiology research suffers from a lack of experimental data on motor axon and dendritic membrane properties relative to somatic properties, due to the relative ease with which somatic properties are recorded. For example, some factors which affect MN repetitive firing can be determined fairly easily in the soma, such as inter-spike variations in voltage spike threshold (i.e. Powers and Binder 2001). Direct recordings such as these are not routinely made in axons or dendrites, although simultaneous measurements of the membrane voltages across all three neuronal compartments are highly desirable in understanding whole neuron behavior. Some axon and dendritic properties are just too difficult to measure experimentally, and alternative methods such as modeling are necessary to elucidate these. But wherever possible, experimental strategies are advantageous in revealing new information, as is historically evident, and this information can be used to advance ideas about neurons. For example, the mechanisms contributing to the greater "repetitiousness" of firing observed in sensory compared to motor fibers remained unclear for a number of decades (Bostock 1995). But in an experiment that used a new *in vivo* electrophysiological technique, it was evident that after ischaemia, sensory axons

can accommodate better to hyperpolarizing currents than motor axons, giving rise to new evidence of differences in inward rectification between the two types of axons (Bostock *et al.* 1994). In addition to highlighting functional differences between axons, this study supplied impetus for further research on the differences between sensory and motor axons (see Lin *et al.* 2002, Kiernan *et al.* 2004, Howells *et al.* 2012).

The present study used the same *in vivo* electrophysiological approach used by the Bostock *et al.* (1994) investigation of sensory and motor axon differences. This approach is AET, and it has been used extensively in recent years to study sensory and motor axons in animal models as well as in humans, in both normal and pathophysiological conditions (Krishnan *et al.* 2009). AET was used in our study to provide an initial estimate of how motor axon bioelectric properties may differ between two populations of motor axons in rats, those innervating TA axons and those innervating SOL axons, which differ in their daily activity patterns (Hennig and Lomo 1985, Gorassini *et al.* 2000). These two populations also differ in the mean values of several MN somatic properties (Kernell 2006). In addition, only 6% of motor units in rat TA are the slow (S) motor unit type, while 94% of the motor units are a fast (fast fatigable or fast fatigue-resistant) motor unit type, as based on a modified version of Burke's criteria originally used to distinguish motor units in the cat hindlimb (Totosy de Zepetnek *et al.* 1992). On the other hand, 80% of the motor units in rat SOL are the slow motor unit type and 20% are the fast motor unit type (Gillespie *et al.* 1987). Also, the TA muscle fiber composition is only 0.3% myosin heavy chain type I and 99.7% myosin

heavy chain types IIa/IId/IIb. The SOL muscle fiber composition is 93% myosin heavy chain type I and 7% myosin heavy chain type IIa (Staron *et al.* 1999). This indicates that both the contractile and immuno-histochemical properties of these muscles are drastically different, which is believed to originate partly because of differences in the daily activity patterns of these muscles (i.e. Gorassini *et al.* 2000, which gives a specific example of different activity patterns in TA and SOL motor units during rat locomotion). Despite the fact that both axon populations contain more than one motor unit type, differences between TA and SOL axons are sufficient for revealing general differences in the axon bioelectric properties of fast and slow motor units. The method of AET offers additional insight into motor axon variations than what is available through traditional measurements such as action potential amplitude or CV.

1.3 Clinical Relevance

Since AET can avoid limitations inherent in other techniques used to examine axon bioelectric properties, it has proved advantageous as a method for investigating the pathophysiology of several diseases which can affect peripheral nerves: ALS, diabetic neuropathy, chemotherapy-induced neurotoxicity, and inherited demyelinating neuropathies (Krishnan *et al.* 2009). However, there is a major assumption in AET which is addressed in this thesis - that only subtle differences exist between motor axons. If this assumption is incorrect, then it is possible that some clinical knowledge espoused by AET will need to be revised. It is well known that MN and axon abnormalities are present in ALS patients, and

that there are significant differences between ALS patients and controls in a number of AET measures (Krishnan *et al*. 2009). However, AET is performed on a group of motor axons within a nerve, complicating the interpretation of results in some scenarios. In ALS, it appears that fast motor units are preferentially degraded more than slow motor units (Dengler *et al.* 1990, Frey *et al.* 2000, Hegedus *et al.* 2008). A selective atrophy of certain motor unit types in ALS patients may mean that some differences in AET measures between healthy controls and ALS patients are actually non-pathological in their origin. In other words, if AET results are significantly different between axons belonging to fast motor units and axons belonging to slow motor units, then a shift in the relative proportion of "fast" and "slow" motor axons will change the AET results for ALS patients. These changes, however, *are separate from any pathological changes* in AET results which may accompany ALS.

The possibility that AET may give differential results for different healthy motor axons may complicate interpretations, but it does not render AET as ineffective for clinical purposes. As mentioned above, conventional nerve conduction studies are limited in scope and mainly assess saltatory conduction along an axon, while AET can measure the activity of multiple ionic conductances over a range of a few milliseconds to a couple hundred milliseconds. Therefore, the processes recorded by AET capture the time-course of the oscillations in excitability which normally occur after a single action potential (Raymond 1979, Bucher and Goaillard 2011). Despite automated AET being relatively new, it has been used to provide insight into the bioelectric properties of motor axons in

healthy people and in peripheral nerve diseases. AET has helped reveal, for instance, changes in voltage-gated sodium (Na^+) channels during acute oxaliplatin-induced neurotoxicity (Krishnan *et al.* 2009). These and other discoveries have proven the worthiness of AET in assessing the function of axon ion channels and electrogenic pumps by *in vivo* and non-invasive methods.

In this thesis, we have sought to better understand the limitations of AET, to know how the motor axon "type" can affect AET results. In addition to the possibility of motor axon type effecting AET, activity levels of individuals may also dramatically change AET results. This indicates that computer modeling or other corrective methods may be needed to eliminate confounding factors in AET. The potential for AET to be used as a diagnostic tool in hospitals is exciting, especially because of the speed with which the 5 main measures of AET can be generated (usually around 10 minutes), the automated manner in which the tests are performed, and the non-invasive nature of the tests. However, more basic science research must first be performed, to build a strong rationale that AET measurements will remain free from confounding factors in a wide variety of patients. This will give clinicians and neurophysiologists greater confidence in this valuable method of peripheral nerve assessment.

In addition to providing basic science research into a potential diagnostic tool in hospitals, this study also has potential implications for neuromuscular rehabilitation programs. The measurements provided by AET in our study clarifies ideas about stimulation parameters for neuromuscular electrical stimulation, which is used as a therapeutic method for maintaining health in

people with a spinal cord injury (SCI), for example. In neuromuscular electrical stimulation literature, it is acknowledged that different widths in the stimulus current can have differential effects on axons, allowing for the possibility of enhanced recruitment of afferent nerve fibers, which can provide a more natural mode of muscle contraction than what is available through stimulation of efferent fibers alone (Bergquist *et al.* 2011). In our study, an examination of differences between fast vs. slow motor axons is undertaken. Perhaps this will allow a new perspective on how the recruitment of one of the two axon groups may be partially enhanced or else partially suppressed, by manipulating the stimulation parameters.

1.4 Unanswered Research Questions Addressed in this Thesis

The manner in which mammalian motor axon bioelectric properties can vary is currently unknown. There have been no studies that have looked at how multiple motor axon bioelectric properties relate or vary with somatic or motor unit properties. This is in contrast with the large number of studies which have investigated how somatic electrical properties relate to motor unit properties or how they co-vary with other somatic properties. The reason is not because motor axon properties are less interesting than somatic properties. Rather, the reason is because there is a bias in contemporary neurophysiology research, which leads to an oversimplification of the role of the motor axon in many studies. There is an allure in thinking of motor axons as analogous to telephone lines, which reliably

and immutably transmit signals in the same way as long as no force damages the line. An alternative is that motor axon bioelectric properties can vary as much and as systematically as MN somatic properties. To begin to address this, we tested the assumption that motor axon properties are not significantly different between TA and SOL axons. The first question addressed in the thesis was thus: *Do significant differences exist in the motor axon bioelectric properties of two groups, fast and slow motor axons, which differ substantially in their activity patterns?* Since they differ in their activity patterns as well as in the properties of their MN somas and muscle fibers, we expect that the axon properties of these two groups are also different.

If the answer to the above question is yes, a second question arises: What are the consequences of these differences between motor axons? Motor axon bioelectric properties may serve to augment the functioning of the motor unit, have little-to-no effect on function, or, perhaps ironically, they may interfere with the function of certain motor unit properties. A secondary spin-off question is: *Do differences between motor axon bioelectric properties reflect adaptations that confer a functional advantage?* Differences in some motor axon bioelectric properties may be functionally advantageous while other properties may be disadvantageous or irrelevant.

A third question is applied to those axon bioelectric properties that we consider relevant to MN physiology: What are the causes of these differences? *Are differences in these properties merely a byproduct of attempts at energy conservation by the cell, or do they represent something more complicated such*

as task-related organization? Certain physiological advantages can arise from the organization and co-variation of MN and motor unit properties (Henneman and Olson 1965, Zengel *et al.* 1985, Kernell 1992). Our ability to speculate upon the advantages supplied to motor units by functional organization at the level of the axon, however, will be limited by the methods employed in our data recordings. Discussion of the properties that may provide a physiological advantage is, then, limited to AET measurements.

Overall, the questions addressed in this thesis focus on physiologically-relevant differences between the two axon groups that were scrutinized**.** Our hypothesis is that differences in motor axon bioelectric properties do exist between fast and slow motor axons, and that these differences arise from differences in Na^+ and K^+ conductances. The reason we believe these specific conductances will be different between the two axon groups is that they have been shown to change at the level of the MN soma, in response to altered activity levels (Gardiner 2006). Specifically, in rats subjected to exercise, the somas of rat tibial MNs show evidence of increased K^+ conductance since there is an increase in AHP amplitude in these somas (Beaumont and Gardiner 2002). In addition, the tibial MN somas of exercised rats also exhibit a faster action potential rise-time (Beaumont and Gardiner 2002). *Hence, we hypothesize that the more active axons, slow motor axons, will show increased outwardly rectifying K^+ conductance as well as increased Na^+ conductance in comparison with fast motor axons.*

1.5 Primary Methodological Approach in the Thesis

1.5.1 Measurements Made by Axon Excitability Testing

Axon excitability testing (AET) measurements were made on left and right hindlimb motor axons in Sprague-Dawley rats, although for the purposes of this thesis we used data from the right hindlimbs only. Specifically, the two groups of axons examined were axons innervating SOL and TA. In AET, 5 main measures are made: **I.** threshold electrotonus (TE), **II.** current-threshold (I/V – this is not to be confused with current-voltage *in vitro* recordings), **III.** recovery cycle (RC), **IV.** rheobase, and **V.** strength-duration time constant (SDTC). Rheobase and SDTC are derived mathematically from physical measurements in a test called charge-duration (Qt). Within each of the 3 main AET measures besides rheobase and SDTC, recordings are made at multiple time intervals and/or multiple conditioning current amplitudes. The most prominent feature shared in each measurement is threshold tracking of a population of axons within a nerve. Threshold tracking is a computer-automated procedure that uses EMG feedback to control the amplitude of a test pulse delivered to axons under a variety of manipulated electrical conditions which are placed on the axon populations. The threshold current represents the test pulse amplitude for every AET measurement, and so these terms are used interchangeably. See sections 2.4.1 and 2.4.2 in this thesis for more information. The various measurements obtained by AET allow for an indirect assessment of the functioning of the ion channels, pumps, and exchangers which collectively give rise to the axon membrane potential (Bostock

et al. 1998, Krishnan *et al.* 2009, Bucher and Goaillard 2011).

1.5.2 Analysis

On 3 of the 5 main AET measures (TE, I/V and RC), we used repeated measures to statistically analyze differences between fast and slow motor axons. In the other two AET measures (rheobase and SDTC), we used paired samples t-tests to compare fast and slow motor axons, since these two measures do not have multiple levels of a within-subjects factor.

In addition, we found evidence that the two anaesthetics used in this study, ketamine/xylazine and sodium pentobarbital, gave differential results in TE and RC. We used independent samples t-tests to compare the effects of the two anaesthetics on certain AET results, since this involved a comparison between two groups of animals which have been given different anaesthetics. Independent samples t-tests were used to compare the anaesthetics since there are no within-subjects factors here. This is in contrast to the analysis of fast vs. slow motor axons, which had one or more within-subjects factors.

1.6 Thesis Organization

This thesis is organized into 5 chapters. The next chapter, "Background and Literature Review", is the longest and includes basic axon concepts studied since the 1930s as well important differences amongst motor axons demonstrated by AET. Chapter 3, "Methods", includes details of the experimental procedures performed in this study as well as information about our statistical analyses.

Chapter 4, “Results”, includes a detailed comparison of fast and slow AET data, as well as serendipitous findings related to our two anaesthetics. Chapter 5, “Discussion”, examines the significance of the results as well as how they fit into the current realms of motor axon and motor unit physiology.

Chapter 2

Background and Literature Review

2.1 Generalizability vs. Specificity of Axon Properties

There is a vast delineation of basic fiber (axon) properties that generalize to axons of the sensory and motor systems in the periphery as well as axons of the central nervous system (Waxman *et al.* 1995). Detailed investigations have been performed on the nerve fibers of many species over the decades, as it was recognized early on that a better understanding of nerve fibers allows a better understanding of motor and sensory functions, and that some fiber characteristics are generalizable across species. Some fiber properties are known to be common across most species, such as the electrical rather than chemical nature of impulse propagation. Other characteristics are evidently unique to specific fibers. The node-internode organization of mammalian myelinated nerve fibers, which is lacking in unmyelinated fibers, is but one example of the specialization of axons

in certain species and tissues. Despite the large variation of some axon properties across species, a Nobel Prize in Physiology/Medicine was awarded in 1944 to Erlanger and Gasser who demonstrated the usefulness of fiber electrical properties in differentiating between "types" of action potentials in peripheral nerve fibers of various species. Also, a 1963 Nobel Prize in Physiology/Medicine was given to Hodgkin and Huxley for their formulation of a mathematical description of the ionic conductances giving rise to the propagated action potential, even though much of their work was based on giant unmyelinated axons of the squid. Thus, although it was widely recognized that some aspects of the axon can differ substantially across the animal kingdom, there were nevertheless some axon properties generalizable across species. It was partly because of this generalizability that Nobel Prize-worthy research could be performed on the axon.

In contemporary neurophysiology, researchers of the axon face a conundrum because of the fact that only some axon properties are generalizable, while other properties must be investigated in many cell types. In the quest to fully understand neurons in a variety of tissues and under a variety of conditions, it is necessary to identify which axon properties can be generalized and which properties must instead be researched across a multiplicity of different cells. A neurophysiologist may study neuron or axon behavior in a particular brain region without ever looking at neurons in the spinal cord or axons in the periphery. Of course, if a certain axon property interesting to researchers (*i.e.* the kinetics of a certain ion channel which effects threshold behaviour, conduction velocity, etc.) is found to vary in some meaningful way across different cells, it is more likely that

related axon properties (*i.e.* the kinetics of other ion channels which also effect threshold behavior, conduction velocity, etc.) will also vary across those same cells, although not necessarily in a way that is correlated to the first property. Future experimental research on axons as well as computational studies modeling axon behavior would both greatly benefit from studies seeking a more comprehensive and systematic understanding of how axon properties vary across different cells. This thesis seeks to aid this process by understanding the differences, if any, in the bioelectric properties between two "types" of motor axons – those innervating TA and those innervating SOL – which are known to differ substantially in their motor unit firing profiles (Gorassini *et al.* 2000), to have different average values in numerous motoneuron (MN) somatic electrical properties (Kernell 2006), and also to have marked differences in the relative number of type-identified muscle fibers/units that they innervate (i.e. for immuno-histochemical evidence see Staron *et al.* 1999; for motor unit mechanical evidence see Gillepsie *et al.* 1987 and Totosy de Zepetnek *et al.* 1992).

2.2 Basic Fiber Electrical Properties

2.2.1 Erlanger and Gasser

Any contemporary discussion of the bioelectric properties of axons owes much to the seminal work of Erlanger and Gasser who, throughout the 1920s and 30s, provided exhaustive accounts of many electrical properties of nerve fibers. Measurements of action potentials, recorded external to the nerve, were made possible in the l920s through the adaptation of the cathode ray oscillograph to

physiology research (Erlanger and Gasser 1937). The threshold for stimulation, propagation speed, and action potential duration and magnitude were the most basic properties dealt with by these two Nobel laureates. Initially, these properties seemed to provide evidence of distinct groups of nerve fibers. Six separate types of compound action potentials (CAPs) were typically visible in the spectrum of potentials produced by a population of fibers within the bullfrog sciatic as well as other nerves. In order of decreasing propagation speed (or increasing stimulation threshold) these potentials were classified as: Aα, Aβ, Aγ, Aδ, B, and C. When analyzing nerve fibers by dividing them into motor and sensory roots, sensory fibers were found to have all of the types of compound potentials, but motor fibers did not have a full complement of types. In the bullfrog sciatic nerve, the propagation speed ranged from 42 to 0.3 m/s. The threshold for stimulation of the least excitable fibers was three hundredfold larger than the most excitable fibers, and the diameters ranged from 1 to 20 μm across all fibers (Erlanger and Gasser 1937). Although the CAPs produced by a group of fibers in a nerve could be organized into distinct "types", the CV (assumed by Erlanger and Gasser to be directly correlated with fiber diameter) and excitability of *single* fibers - all within the same nerve - varied in a continuous manner. In addition, it was found that the action potential amplitudes and durations of single fibers varied in a continuous manner. It was also noted that *individual* action potentials from single nerve fibers could extend across distinct parts of the CAP spectrum (consequently causing ambiguity in their Aα/Aβ/Aγ/Aδ/B/C classification scheme), due to such things as fibers branching to smaller diameters as well as long action potential

durations. These findings led Erlanger and Gasser to assert that their six distinct "types" of CAPs were in fact a pedagogical classification system rather than a functional one. From this, they tentatively concluded that propagation speed provided the most meaningful functional distinction between different single fibers in a mixed nerve (as well as fiber diameter since these two properties were found to be strongly correlated), even though this property was found to vary continuously and therefore argued against the idea of distinct functional "types" of fibers. Erlanger and Gasser found certain other electrical properties to vary with discontinuity, although the functional implications of this was not immediately obvious. This was the case for chronaxie in their investigations (Erlanger and Gasser 1937). However, since these two pioneering researchers found most electrical properties to vary continuously, this was considered to provide strong evidence that there are not distinct "types" of fibers. Such was the case for not only CV and action potential amplitude and duration, but also for fiber excitability, relative refractory period (RRP) and absolute refractory period (Erlanger and Gasser 1937).

2.2.2 The A, B, and C Axon Classification Scheme Today

Despite the fact that there is a continuous variation in several of the electrical properties which underlined Erlanger and Gasser's "types" of potentials in the CAP of a mixed nerve, and despite there being no easy way to separate individual fibers into functionally-distinct classes, the terminology originally employed by Erlanger and Gasser is widely used today. This system of classification, however, is now used either as a means of classifying fibers with different CVs or else to

identify fibers with different anatomical origins or destinations. The exact definition of the terms Aα, Aβ, Aγ, Aδ, B, and C can range substantially, however. Whitwam (1976) proposed that Erlanger and Gasser's A, B, and C fibers should only be applied to efferent fibers. He suggested that Aα should refer to myelinated efferent fibers with CVs between 70 and 120 m/s, Aβ refer to myelinated efferent fibers with CVs between 50 and 70 m/s, Aγ refer to myelinated efferent fibers which project to intrafusal muscle fibers (which have CVs sometimes between 30 and 50 m/s but also in the Aδ range), and Aδ refer to myelinated efferent fibers with CVs under 30 m/s. In this classification proposed by Whitwam, B fibers would refer to any myelinated efferent fiber of the autonomic nervous system, with CVs usually in the same range as Aδ fibers. It was then proposed that the term C fiber refer to unmyelinated efferent fibers of the autonomic nervous system. Under this classification, the term C fiber would be separate from group IV fibers which were originally classified by Lloyd (1943) as unmyelinated afferent fibers travelling in dorsal roots. This terminology proposed by Whitwam has not been universally adopted, and one is to be cautious when interpreting Erlanger and Gasser's A, B, and C designations in the axon literature. For example, Aα fibers can sometimes refer to *any* somatic efferent fiber innervating extrafusal muscle fibers, without any explicit reference to CV (i.e. Gustafsson and Pinter 1984, Gardiner 2006).

2.2.3 Axon Threshold Properties

The early studies by Erlanger and Gasser confirmed that important fiber properties, such as CV, action potential amplitude and duration, etc., varied

continuously and this was believed to argue against the presence of functionally-distinct types of fibers within a mixed nerve. However, many subsequent studies have sought to describe how the many bioelectric properties of nerve fibers vary, in search for the functional implications of axon variation. Of enduring interest to researchers has been axon behavior during or after subthreshold, near-threshold, or suprathreshold stimulation. For example, Erlanger and Blair (1938) found that the fibers of ventral roots accommodated (opposed membrane polarization) better than those of dorsal roots in certain conditions, and so the propensity for "repititiousness" was found to be less in motor vs. sensory fibers after certain stimuli. This observation has been confirmed in later studies (Bostock 1995b), although the complex bioelectric differences between motor and sensory axons are still being investigated (Krarup and Moldovan 2012, Howells *et al*. 2012, Nodera and Rutkove 2012). The threshold and accommodative properties of fibers continue to be of great clinical interest today because these properties can be used to infer the underlying pathophysiology in various disorders of peripheral nerves. Finally, in the context of the present study, the threshold and accommodative properties of fibers can be used to uncover the functioning of axon ion channels, pumps, and exchangers (Krishnan *et al.* 2009).

2.2.4 Conduction Velocity

Axon diameter and conduction velocity (CV) have remained fundamental axon properties that have been useful in neurophysiology research over many years (i.e. Erlanger and Gasser 1937, Hursh 1939, Rushton 1951, McPhedran *et al*. 1965, Moore *et al.* 1978, Carp *et al.* 2003, Barry *et al.* 2012). Various axon properties

such as passive (membrane resistance, axial resistance) and active (voltage-gated Na^+ channel density/distribution and kinetics) properties can affect CV. Axon diameter is usually thought to exert a substantially greater influence on CV than other axon properties in normal, healthy mammalian axons. Therefore, barring any large developmental or pathological changes to axon morphology, it is generally assumed that the CV of individual axons remains relatively stable over time. In addition, CV has been assumed to correlate with MN somatic size (i.e. Henneman *et al.* 1965a), although at higher values CV is mostly uncorrelated to somatic size as estimated by cell capacitance (Gustafsson and Pinter 1984). Importantly, the CV of individual adult axons in different mammalian species have actually been shown to change over short time scales (*i.e.* over milliseconds, seconds, or minutes; see Bullock 1951, Swadlow *et al.* 1980, Baker *et al.* 1987, Thalhammer *et al.* 1994) as well as over long time scales (*i.e.* over days or months; see Swadlow 1982, Carp *et al.* 1994, Munson *et al.* 1997, Carp *et al.* 2001, Beaumont and Gardiner 2002). It appears that, in general, increased activity levels (from chronic low frequency stimulation, exercise training over 6 weeks or more, etc.) tend to cause slower CVs. Conversely, changes incurred after chronic spinal lesions can both increase and decrease CV. For example, axons innervating cat medial gastrocnemius and lateral gastrocnemius have a significantly lowered CV following a spinal cord lesion, while axons innervating cat SOL have a significantly increased CV (Hochman and McCrea 1994a, Munson *et al.* 1986, Cope *et al.* 1986).

One laboratory has specifically sought to understand the underlying causes

of motor axon CV changes associated with the conditioning of H-reflexes of primates and rats through rewards which are contingent upon reflex amplitude. This laboratory has found a strong inverse relationship between CV and the action potential threshold at the axon (Carp *et al.* 2003). The authors have argued that the simplest explanation for the depolarizing shift in firing threshold which accompanies a decreased CV, seen in triceps surae motor axons of animals rewarded for producing lower SOL H-reflex amplitudes (Carp *et al.* 1994, 2001), is a slowing of voltage-gated Na^+ channel kinetics (Carp *et al.* 2003). Modeling evidence supports the notion that CV is sensitive to voltage-gated Na^+ channel behavior (Moore *et al.* 1978), with overall decreases in nodal Na^+ conductance corresponding to a decreased CV. Recently, a study using gene replacement to increase axon diameter through elongated neurofilament medium C termini in mice found that CV did not increase along with axon diameter, and modeling evidence indicated this was likely because myelin thickness didn't change in proportion to axon diameter (Barry *et al.* 2012). Taken together, these studies attest that CV, a function of both active and passive axon properties, can undergo changes which are independent of changes to axon diameter. Furthermore, in addition to showing activity-dependent changes in CV, these studies endorse the possibility that motor axons can “adapt” to activity by changing their bioelectric properties, perhaps even changing properties which can be measured by AET.

2.3 Activity-Dependent Changes in Motoneuron (Soma) and Muscle Properties

The notions of motor axon plasticity is strengthened when considering the plasticity of MN somas and their innervated muscle fibers. Although there are a large number of biochemical changes which occur in both the soma and muscle in response to altered activity, we will restrict this discussion to changes in somatic electrophysiology or muscle fiber mechanical properties. Generally, a chronic increase in neuromuscular activity, through treatments such as treadmill training or electrical stimulation, leads to a transition to a more 'slow' phenotype in the MN soma as well as in the innervated muscle fibers. In these treatments, somatic electrical properties become more like those of small somas which typically have a larger AHP duration, lower rheobase, and larger input resistance. This phenotype can be considered akin to Henneman's "small and excitable" MNs. A chronic increase in activity also generally leads to a more "slow" phenotype in muscle fibers, so that many properties of the trained muscle shift towards the direction of a slow muscle such as SOL and away from a fast muscle such as TA. Properties of the individual motor units in these treatments shift towards values typical of Burke's slow motor unit type. Indeed, the coordination of MN and muscle properties (whole muscle and/or individual motor unit properties) which is seen under normal conditions (i.e. Zengel *et al.* 1985) tends to be maintained after chronic alterations to activity in the form of spinal transections (Cope *et al.* 1986, Munson *et al.* 1986). Also, coordination between somatic and muscle properties is maintained after chronic 20 Hz electrical stimulation, delivered every 5 out of

10 seconds to self-reinnervated medial gastrocnemius motor units (Gordon *et al.* 2004).

The coordinated shift of MN and muscle properties after altered activity levels is demonstrated clearly in chronic electrical stimulation experiments, which are able to induce changes to neuromuscular activity in a quantifiable way. For example, there are parallel changes in MN and motor properties after chronic low frequency stimulation, at a rate of 20 Hz delivered for 2.5 seconds every 5 seconds (a 50% duty cycle), of the healthy cat medial gastrocnemius (Munson *et al*. 1997, Gordon *et al.* 1997). For the MNs, chronic low frequency stimulation led towards a more slow phenotype, so there was a significant decrease in rheobase and a significant increase in AHP duration and input resistance (Munson *et al.* 1997). The changes in somatic membrane properties reported in the study by Munson *et al.* were consistent with overall changes at the level of the motor unit towards a more slow phenotype. These changes were a decline in force, a slowing of twitch speed, and an increase in the Burke fatigue index - changes which are consistent with the muscle fibers becoming more like Burke's slow-type motor unit (Gordon *et al.* 1997, the companion paper to the Munson *et al.* 1997 paper). In general, chronic increases in neuromuscular activity via chronic stimulation induces an increase in the fatigue resistance of the muscle and a decrease in contractile speed (Gordon *et al.* 1997, Kernell *et al.* 1987a, 1987b) plus increased MN excitability (Munson *et al.* 1997). The effects of decreased neuromuscular activity are generally opposite to these effects of increased activity, so that MN somatic and muscle properties change to a more “fast”

phenotype after decreased activity (i.e. through spinal transection, hindlimb unweighting, etc.).

The use of chronic stimulation in studying MN and muscle plasticity has two main advantages over other models of altered activity such as exercise: the change in activity can be readily quantified through strict control of the stimulation parameters (Kernell 2006) and there is synchronous activation of motor units across a wide range of recruitment thresholds thereby producing increased activity in less excitable, large fatigable motor units (Pette 2002). These large fatigable units are probably relatively less active in exercise treatments than in chronic stimulation treatments. The question has arisen, however, as to the relative importance of different stimulation parameters, such as frequency and total daily duration of activity, in influencing MN and muscle phenotypes. For instance, does the shift towards a more slow phenotype arise because of an application of low frequency stimulation? Or, are long durations of activity such as stimulation delivered for a total of 12 hours each day more important in shifting MN and muscles towards a slower phenotype? In comparison to chronic low frequency stimulation, chronic high frequency stimulation (i.e. 40 vs. 10 Hz stimulation delivered for a total of 12 hours each day) *does not* consistently lead to a more fast phenotype in regards to muscle contractile speed (Kernell 2006, Gordon *et al.* 1997). In addition, the twitch speed of peroneus longus muscle *is not* shifted to a faster phenotype by superimposing small daily amounts of high frequency stimulation (100 Hz) on top of large amounts of low frequency stimulation (Kernell *et al.* 1987a, 1987b) or

through delivery of small daily amounts of high frequency stimulation during a recovery period that follows chronic low frequency stimulation (Kernell and Erbeek 1991). In addition, both fast (40 Hz) and slow (10 Hz) rates of chronic stimulation producing activity for more than 50% of each day can convert all cat peroneal longus fibers to myosin heavy chain type I (Donselaar *et al.* 1987) and which also produce similar effects on contractile speed and endurance (Eerbeek *et al.* 1984).

Given the data above on chronic stimulation studies, this would seem to suggest that a fairly wide range of stimulation frequencies can have a uniform effect on muscle contractile properties. However, different rates of stimulation can produce differences in succinate dehydrogenase staining (Donselaar *et al.* 1987) as well as differences in contractile force in muscles. Indeed, higher stimulation frequencies generally produce a larger contractile force (see Eerbeek *et al.* 1984, Kernell *et al.* 1987a, Westgaard and Lomo 1988). In addition, the slowing of rat soleus twitch speed caused by chronic low frequency stimulation can be partly counteracted by superimposing short bursts of high frequency stimulation on top of the low frequency stimulation (Westgaard and Lomo 1988). Therefore, it appears that stimulation frequency is an important parameter that mostly influences contractile force, but which also influences twitch speed in some muscles. Thus, stimulation frequency influences twitch speed in rat extensor digitorum longus and soleus but not in cat peroneus longus (Westgaard and Lomo 1988, Kernell *et al.* 1987a). Total daily duration of stimulation (or the number of impulses) greatly influences fiber fatigability, but it also seems to have

an effect on twitch speed and possibly contractile force as well (Westgaard and Lomo 1988, Kernell *et al.* 1987a, Kernell 2006). It is important to note that the relative effects of these stimulation parameters depend not only on the particular muscle under investigation but on the species as well. In addition to the influence of pulse rate and total daily duration of activity, it is possible that other stimulation parameters, such as intervals between bursts, may also influence muscle properties and perhaps MN properties as well (Kernell 2006).

Exercise treatments have also been used to study activity-dependent plasticity of MN and muscle fibers. In line with findings from chronic stimulation studies, endurance exercise training promotes a shift in muscle properties towards a slow phenotype. This is exemplified by a shift towards a higher proportion of the fatigue-resistant muscle fibers containing myosin heavy chain type I and/or type IIa seen after many longitudinal studies of endurance exercise training (MacIntosh *et al.* 2006). Also, information obtained from motor unit firing patterns in the first dorsal interosseus indicates there is larger excitability and a larger AHP amplitude in MN somas from the dominant compared to the non-dominant hand (average motor unit firing rate, initial firing rate, and recruitment threshold are all lower in the dominant hand, presumably because of increased activity – from Adam *et al.* 1998). In comparison with chronic electrical stimulation, exercise treatments may not see as large of a change towards the slow phenotype, probably since the total daily duration of activity in exercise treatments is less. For example, low frequency chronic stimulation is often delivered for more than 30% of the day and so these stimulated units are active

for more than 7.2 hours each day. However, in normal daily movements, even the most active units are usually active for less than 30% of the day (see Kernel 2006, Hennig and Lomo 1985). Also, the results of exercise studies are more difficult to interpret since asynchronous activation of motor units during exercise leads to differential activity changes across a MN pool. Moreover, studies in rat tibial MNs involving either voluntary spontaneous exercise or forced endurance exercise have shown that MNs can be sensitive to the type of exercise. In rat tibial MNs, voluntary spontaneous exercise does not change AHP duration but instead causes a significantly larger AHP amplitude as well as hyperpolarization of the resting membrane potential and hyperpolarization of the voltage threshold. It has been suggested that these findings from rats subjected to voluntary exercise indicate decreased persistent inward Na^+ currents as well as a lower overall excitability during prolonged firing in tibial MN somas (Beaumont and Gardiner 2002, 2003, Gardiner *et al.* 2006).

A chronic decrease in activity, or disuse, can be simulated through experimental interventions such as a spinal transection, spinal isolation (transection plus deafferentation), and hindlimb unweighting. Each of these treatments produces their own unique effect on MN soma and muscle fiber properties (i.e. compare Cormery *et al.* 2005 with Button *et al.* 2008). However, as mentioned above, generally these treatments lead to a shift towards a more fast phenotype (Kernell 2006, Gardiner 2006). For example, hindlimb unweighting in rat tibial MNs produces a significantly larger rheobase, lower AHP amplitude, and a shift to the right in the frequency-current relationship (Cormery *et al.* 2005).

Spinal transection in cats can lead to a statistically significant larger proportion of extensor hindlimb motor units having a "fast" phenotype, determined using MN electrophysiological (Hochman and McCrea 1994b) and muscle unit mechanical criteria (Cope *et al.* 1986, Munson *et al.* 1986).

The causes underlying disuse-related changes are difficult to interpret, due to the fact that the alteration in activity is usually not well quantified. In hindlimb unweighting studies the MNs are still free to cause activation of their innervated muscles despite there no longer being any benefit for posture. In the hindlimb unweighting study cited above (Cormery *et al.* 2005), no data was provided in this article with regard to the activity of the motor units (i.e. through EMG analysis) throughout the treatment of hindlimb unweighting. Also, the complicated effects of spasticity which can be associated with spinal transection (i.e. Alaimo *et al.* 1984, Bennett *et al.* 2004) makes it difficult to quantify the changes to activity in this disuse model. In summary, it is possible that the differential effects of hindlimb unweighting and spinal transection, on muscle and MN properties, are simply due to different changes in neuromuscular activity patterns. Although hindlimb unweighting and spinal transection/isolation have helped increase knowledge about MN and muscle plasticity, findings from these studies need to be interpreted in light of other studies. This will help to more fully elucidate all of the mechanisms, as well as the consequences, involved with plasticity of the neuromuscular system. In summary, although all kinds of treatments are useful for increasing knowledge about the plasticity of MNs and muscle, chronic electrical stimulation has traditionally been the most common method since this

treatment is able to produce uniform activity patterns that can be strictly controlled. Finally, it is worth noting that recent studies have demonstrated that the plasticity of MN somatic bioelectric properties likely reflects a chronic change to ionic conductance(s) (Gardiner *et al.* 2005, Cormery *et al.* 2005, MacDonell *et al.* 2012), which is of relevance to this thesis since AET is sensitive to changes in certain axon ionic conductances. Therefore, these findings of activity-dependent changes to somatic ionic conductances may indicate the possibility of activity-dependent changes in axon ionic conductances.

2.4 Evidence of Variation in Motor Axon Properties: Axon Excitability Testing

We have briefly reviewed some important studies which have demonstrated activity-dependent plasticity in the MN soma, the muscle unit, and axon CV. It seems feasible to postulate that functionally-relevant properties of the motor axon (in addition to CV), previously overlooked because of limitations in methods, also exhibit plasticity. AET is one method which can overcome the traditional limitations faced when studying the electrophysiology of axons. AET provides a rapid (i.e. under 20 min) *in vivo* assessment of multiple axon bioelectric properties. These axon bioelectric properties are shaped by underlying processes such as ion channel function, Na^+/K^+ pumps, and membrane potential (Bostock *et al.* 1998, Krishnan *et al.* 2009; see below), and therefore AET provides an indirect examination of active properties of the axon. In addition, it has been suggested that AET may provide a sort of remote glimpse into overall MN functioning and

metabolic activities affecting the axon (Jankelowitz *et al.* 2007, Boland *et al.* 2009; see below). In SCI patients there have been recent suggestions that AET might also detect extrasynaptic processes which affect the axon, even in the absence of systemic metabolism abnormalities (von Bartheld 2004, Boland *et al.* 2009, Boland *et al*. 2011). Moreover, AET is relatively insensitive to CV and myelination of an axon (in comparison to conventional nerve conduction studies), and instead scrutinizes axon properties at a single locus along the nerve (Bostock *et al.* 1998). Therefore, AET complements conventional nerve conduction studies which are sensitive to CV, myelination, and the the number of axons which are able to conduct action potentials in response to electrical stimulation. The powerful inferential capabilities of AET have been employed in a variety of pathologies affecting peripheral nerve function: diabetic neuropathy, oxaliplatin-induced neuropathy, ALS, and even multiple sclerosis (Ng *et al.* 2008, Krishnan *et al.* 2009). In addition, this electrophysiological tool for examining peripheral axons has been utilized in the investigation of motor-sensory differences (Bostock *et al*. 1994, Howells *et al.* 2012) as well as differences between motor axons of different thresholds and between motor axons supplying different muscles (see table 2.1 below and associated discussion). Given the large range of conditions with which AET has detected significant differences in axon bioelectric properties, in pathological as well as normal conditions, it is surprising that this method has not yet been used to directly examine the possible effects of altered activity on peripheral motor axons. This is doubly surprising considering the ample documentation of plasticity in MN somatic bioelectric properties in

response to exercise, hindlimb unweighting, and spinal cord transection, as well as other conditions of altered activity (see section 2.3).

2.4.1 Principles of Threshold Tracking

The AET measures in this study are made possible through threshold tracking. Threshold tracking is a technique which uses a target compound muscle action potential (CMAP) to control stimulation current applied to a nerve, or axon group, innervating the muscle. The target CMAP then provides a method of "constant response", where the error in the observed muscle response vs. the target muscle response serves as feedback control of the test current amplitude. Using this method, numerous kinds of estimations of axon excitability can be made, always determined at the same size of muscle response. The method of constant response allows for reliable tracking even when threshold increases by more than 200% (Bostock *et al.* 1998). Also, the target response waveforms are nearly identical to each other in every successful threshold estimation in this method (Bostock *et al.* 1998), although the group of axons being examined by threshold tracking can change between each stimuli (Mori *et al.* 2010). This happens since threshold changes induced by a conditioning stimulus are often greater than the threshold differences between many axons at rest. In the present study, we have examined axons innervating TA and SOL, and therefore changes in recruitment order throughout threshold tracking are unlikely to complicate interpretation of our results since TA axons can be assumed to be uniformly from fast motor units while SOL axons are almost uniformly from slow motor units.

Threshold Tracking Feedback Loop

We have used the method of constant response in the threshold tracking used in this study. Below we provide the generic feedback loop which captures the threshold tracking methods used in all of the 5 main AET measures (Fig 2.1). The test pulse always has a duration of 1.0 ms in the main AET measures except rheobase and SDTC (see section 2.4.2.5 for more details). Specifically, the test pulse refers to the amplitude of a *conditioned* test pulse which seeks to produce a muscle response within ± 7.5% of the target CMAP. The target CMAP is where the stimulus-response (SR) curve is steepest (Fig 2.2), which is normally near 40% of a maximal CMAP. Unconditioned current refers to the *unconditioned* current amplitude required to produce the target CMAP. The amplitude of the test pulse varies across the main AET measures, in contrast to the amplitude of the unconditioned current which is virtually constant across all of the AET measures.

Stepping through the flowchart Fig 2.1, we see that each and every new data measurement starts with a new conditioning-test pulse arrangement. In the case of threshold electrotonus (TE), the program starts to record a new data value by (a) changing the size of the submaximal conditioning pulse or (b) changing the delay between the conditioning and test pulses. In current-threshold (I/V), the program changes the size of the submaximal conditioning pulse in 10% increments from a depolarizing pulse of +50% to a hyperpolarizing pulse of -100%. In the recovery cycle (RC), the program simply changes the delay between a supramaximal conditioning pulse and the test pulse and then seeks to achieve the target CMAP. Finally, in the charge-duration recordings (which

determines values for 2 main AET measures, rheobase and strength-duration time constant), the program obtains single values by varying the duration of the test pulse between 1.0 ms and 0.2 ms.

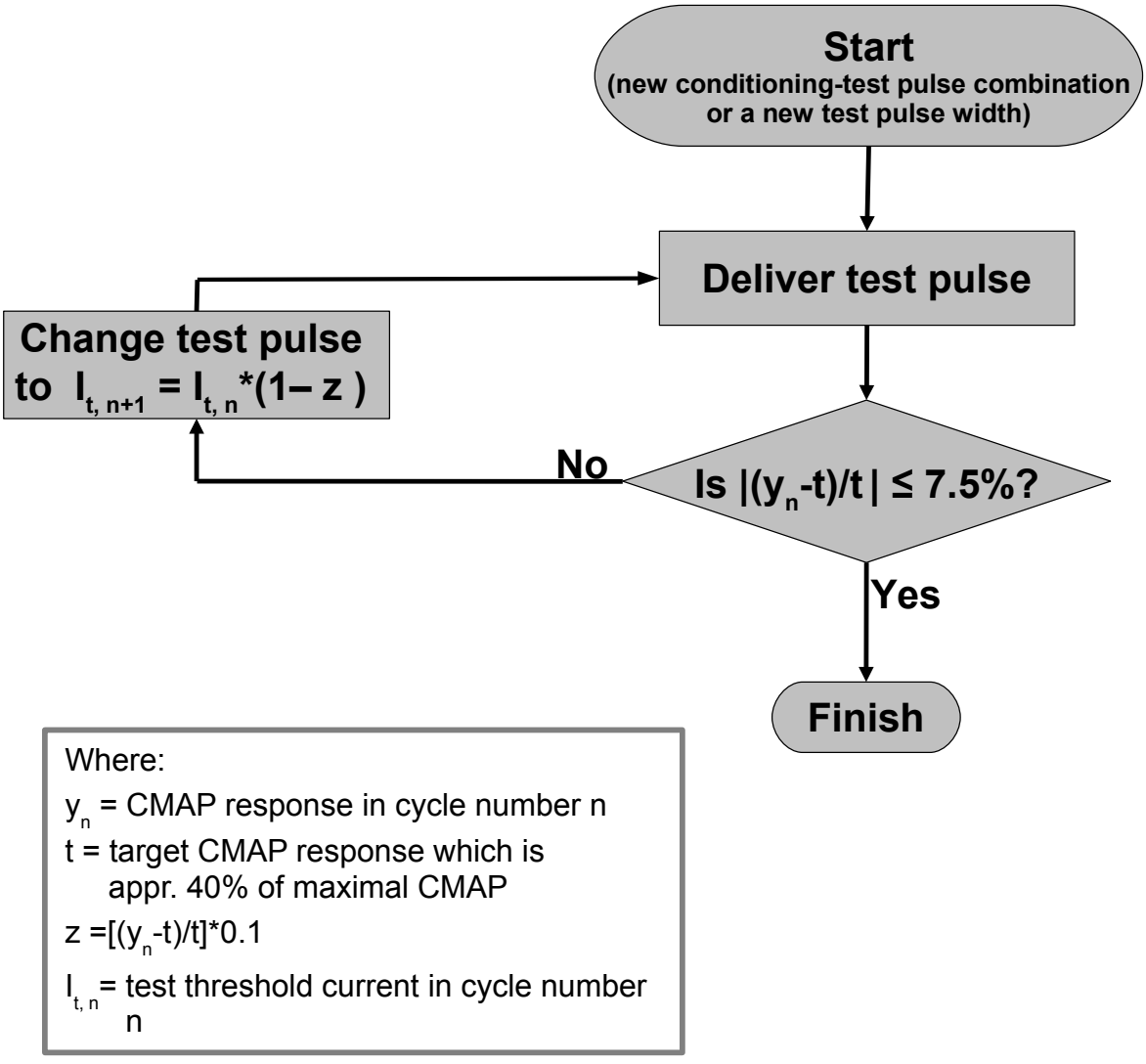


Figure 2.1. This depiction of threshold tracking shows the standardized method of data collection for the 5 main AET measures. It is important to note that the magnitude of every data point collected is simply the test pulse amplitude that is recorded at "Finish", in all of the main AET measures except rheobase and SDTC (these 2 measures are actually determined by the linear relationship between 5 data points – see section 2.4.2.5).

Every cycle in the feedback loop is 800 ms long, which means that the

interval between consecutive test pulses is 800 ms. As previously mentioned, the test pulse seeks to achieve the target muscle response within a certain level of error. The error between the observed and target CMAPs cannot be greater than 7.5%. This is reflected in the equation:

$$(y_n - t)/t \leq 7.5\%$$

where y_n refers to the observed muscle response in cycle n, and t is the target muscle response (CMAP). When the error is greater than 7.5%, the program alters the size of the test pulse so that if the muscle response is too small the program increases the test pulse amplitude but if the muscle response is too large the program decreases the test pulse amplitude. This is reflected in the equation:

$$I_{t,\, n+1} = I_{t,\, n} - z$$

where $I_{t,\, n+1}$ is the new test pulse amplitude that will be delivered in the next cycle, number n+1, $I_{t,\, n}$ is the previous test pulse amplitude in cycle n, and z is the amount by which the test pulse is altered. Essentially, the direction and size of z is dependent on the direction and size of the error between the observed and target CMAPs, with a greater magnitude of error resulting in a larger adjustment of the test pulse amplitude. This method of test pulse adjustment is called "proportional tracking", and it generally reduces the number of cycles required before the program can "finish" recording the data value. The data value is really the test pulse amplitude. By contrast, the method of "step-size tracking" alters the test pulse amplitude by fixed amounts which are independent of the error between the observed and target CMAP. Therefore, in proportional tracking the correct amplitude for the test threshold current is found more quickly than in step-size

tracking, due to a reduction in the overall number of test pulses (cycles) needed before hitting the target muscle response.

In this thesis, each of the 5 main AET measure obtained through threshold tracking have one independent variable in addition to the "axon group" independent variable. The second independent variable is "delay" in the four TE variants (there are two TEd and two TEh tests) and also in RC, "conditioning strength" in I/V, and "pulse duration" in rheobase and SDTC. Rheobase and SDTC are unique in that these tests use multiple physical measurements to obtain a *single* measurement.

2.4.2 Principles of Axon Excitability Testing

2.4.2.1 Stimulus-Response Curve

At the very beginning of threshold tracking, before any AET measurements are made, a stimulus-response (SR) curve must be generated (Fig 2.2). A maximal CMAP is determined at the very start of the experimental data collection, then individual CMAP responses and their associated stimulation current amplitudes are determined incrementally from 2% to 98% of the maximal CMAP. All of the AET measurements are dependent on the results from the SR curve generated from this, since threshold tracking utilizes the slope of the SR curve to pinpoint exactly what size of CMAP will be the target. The slope that is chosen is determined from the point of the SR curve where it is steepest (between 25 and 50% of maximal CMAP). A CMAP around 40% of maximal ($CMAP_{40\%}$) seems to be often where the SR curve is the steepest.

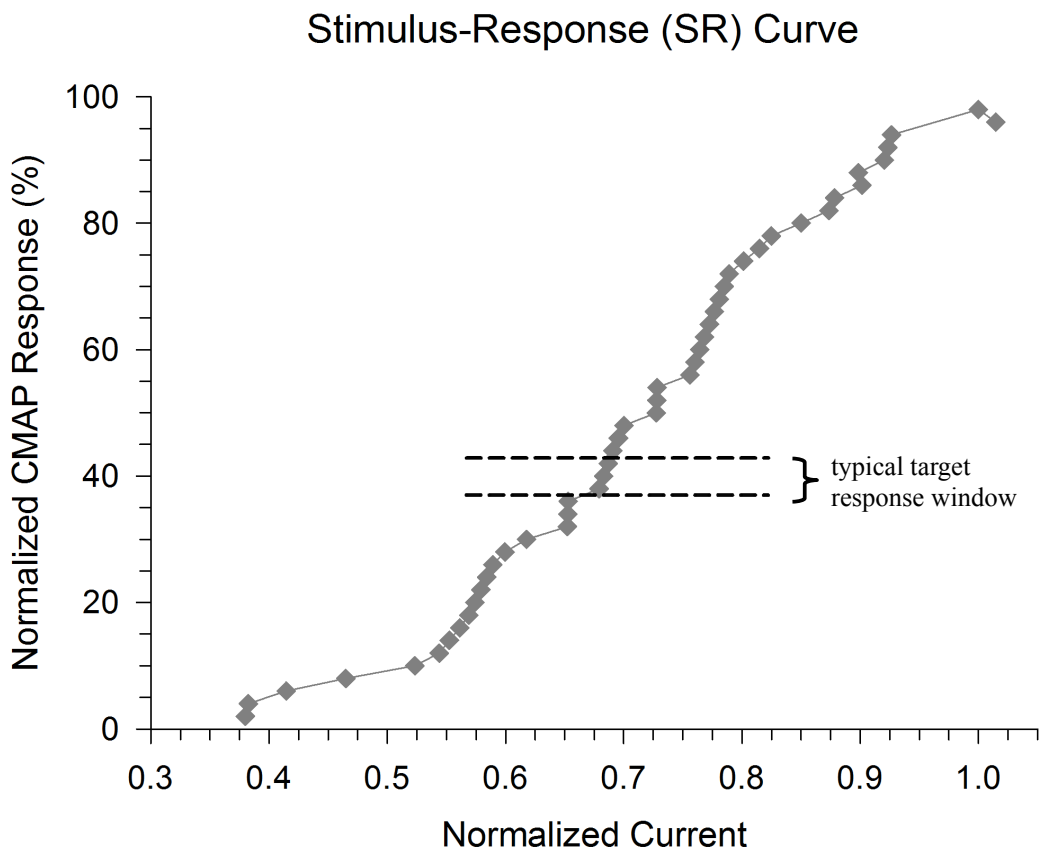


Figure 2.2. Representative plot of a normalized SR curve. The SR curve is often sigmoidal in shape. This curve is generated in each rat before any AET measurements are made, as it is a crucial component of threshold tracking and determines the optimal target response, usually near 40% of the maximal CMAP, which is "tracked" throughout all recordings.

In some preparations, the slope of the SR curve is too steep to use proportional tracking, and so in these cases step-size tracking is used instead. It should be noted that changing from proportional to step-size tracking does not affect any AET measurements, since the only difference between these two methods is the number of test pulses that were needed before achieving the target CMAP in three consecutive trials. Therefore, the target CMAP is the same in proportional and step-size tracking.

2.4.2.2 Threshold Electrotonus

In threshold electrotonus (TE), a submaximal conditioning pulse with a duration of 100 ms is applied to the axons, and the interval between the onset of this conditioning current and the onset of the test pulse is incrementally increased from 0 to 200 ms (onset occurs at x =10 ms and offset at x =110 ms). None of the delay increments are larger than 10 ms. For TEd (depolarizing TE tests with the conditioning current set to either +40% or +20% of the current required to produce the target CMAP), there are 27 data points collected during the 200 ms recording window (Fig 2.3). For TEh (hyperpolarizing TE tests with the conditioning current set to either -40% or -20% of the current required to produce the target CMAP) there are 25 data points collected. TE is displayed in such a way that increases in axon excitability (increases in threshold reduction) are plotted above the x-axis. Decreases in excitability are plotted below the x-axis. In TEd, threshold is rapidly reduced (due to rapid depolarization of the axons) in the first ~2 milliseconds, which is termed the F phase. The F phase is due mainly to passive spreading of charge to axon nodes. After the F phase, excitability usually increases at a slower rate for another few milliseconds, which is termed the S1 phase and is due to the spread of charge to the axon internode (Krishnan *et al.* 2009). The S2 phase (also called S2 accommodation) then occurs between approximately 20 ms and 110 ms in the TEd tests, where the axon excitability is decreased via the activation of nodal slow K^+ channels. Therefore, in the S2 phase of TEd there is a downward "push" of the waveform toward baseline which lasts during most of the 100 ms depolarizing conditioning current. Finally, after

the offset of the depolarizing conditioning current at delays of 110 ms and beyond, excitability drops below baseline and so the TEd line goes below the x-axis (i.e. there is a decrease in axon excitability or an increase in threshold). This period of decreased excitability is called TEd undershoot and is due to hyperpolarization caused by slow deactivation of slow K^+ channels.

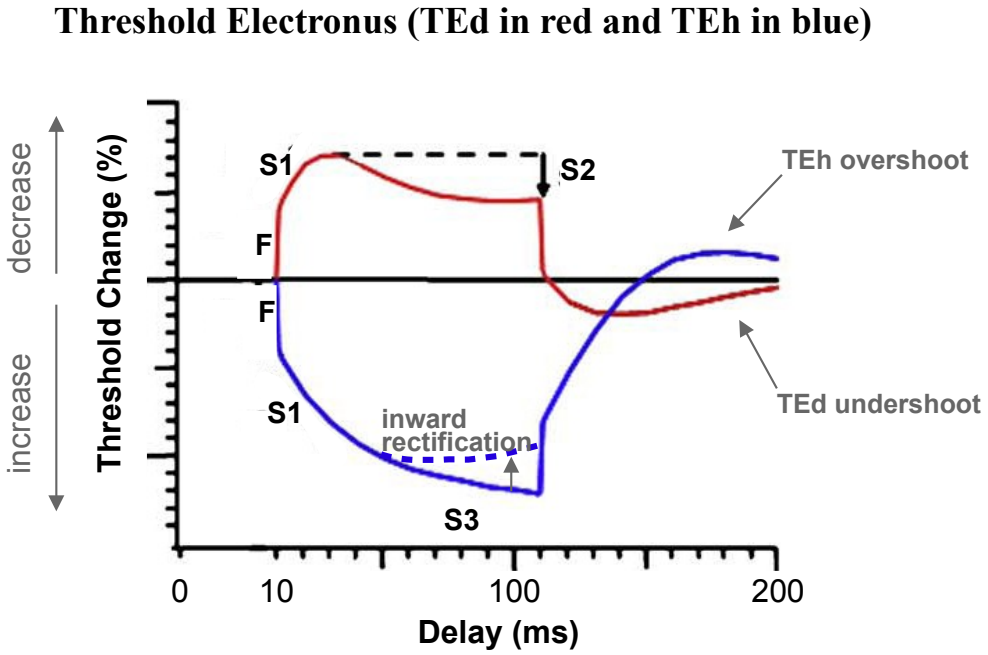


Figure 2.3. Representative plot of TE, in which long-lasting depolarizing conditions (TEd) or hyperpolarizing conditions (TEh) are applied to axons. The dashed blue line found within S3 shows the specific change in the TEh plot which occurs in axons with greater inward rectification. Threshold decreases are plotted above the x-axis, with increases in threshold plotted below the x-axis. Adapted from Krishnan *et al.* (2009).

In the two TEh tests, there are also F and S1 phases which have approximately the same time-course as the corresponding phases in the two TEd tests. Unlike the S1 phase in TEd, however, the S1 phase in TEh does not act to limit polarization since K^+ channels are deactivated under hyperpolarizing conditions. An S3 phase occurs in TEh also (as opposed to the S2 phase in TEd), and this is due to activation of the hyperpolarization-activated inwardly rectifying

cation conductance (I_H) rather than activation of outwardly rectifying K^+ conductances. In the S3 phase, inward rectification increases (axon excitability also increases) when I_H activity increases. Finally, after the offset of the conditioning current at delays beyond ~115 to 120 ms, axon excitability goes above baseline. This final phase of the TEh waveform is called TEh overshoot and it is a result of the coupling of (i) slow deactivation of $I_{H,}$ and (ii) activation of outwardly rectifying slow K^+ conductances (Krishnan *et al.* 2009).

TE has been an important method used for the study of a variety of peripheral axon abnormalities seen in ALS, diabetic neuropathy, and oxaliplatin-induced neuropathy (Krishnan *et al.* 2009). Due to the long duration of the conditioning pulse, TE helps assess internodal properties of the axon. Also, TE provides an indirect examination of the function of three rectifying currents: slow K^+ conductance, I_H , and fast K^+ conductance as well (Baker *et al.* 1987, Lin *et al.* 2002, Jankelowitz *et al*. 2007, Krishnan *et al.* 2009). Finally, TE is sensitive to membrane potential. Membrane depolarization generally results in a "fanned-in" TE waveform (where axon excitability is closer to baseline across all delays, in all TE tests). Conversely, membrane hyperpolarization generally results in a "fanned-out" TE waveform (where axon excitability is further from baseline across all delays, in all TE tests).

2.4.2.3 Current-Threshold

Current-threshold (I/V) is similar to TE in that a long submaximal conditioning pulse is applied to the axons. However, here the test pulse is given at a fixed delay, at the very end of a 200 ms-long conditioning pulse. The factor that varies

in I/V, then, is the size of the conditioning pulse. I/V measurements complement TE quite well. Hyperpolarizing I/V (data on the left of the y-axis in Fig 2.4)

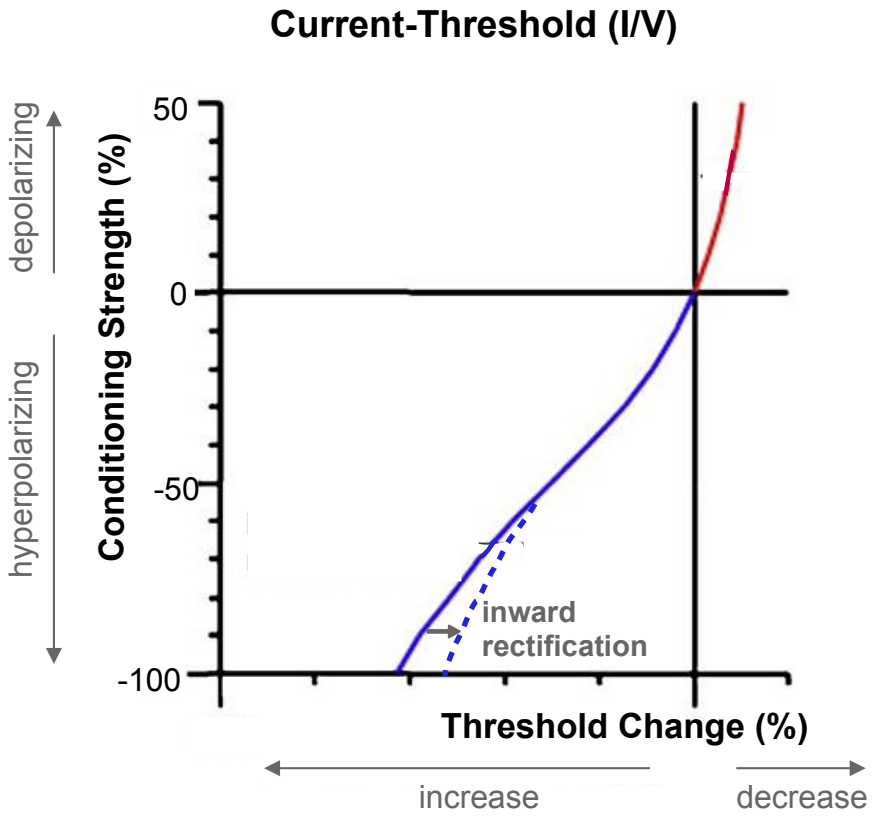


Figure 2.4. A representative plot of current-threshold (I/V). Note the reversal of the axes, in which the independent variable is actually placed on the y-axis. The red line in the top right quadrant represents data obtained from depolarizing conditioning currents while the blue line in the bottom left quadrant is from hyperpolarizing conditioning currents. Inward rectification is the most important feature of I/V. Greater activity in the axon ionic conductance "I_H" results in greater inward rectification, which pushes the blue line towards baseline (towards the right) at strong hyperpolarizing conditioning currents. This is indicated by the dashed blue line. Adapted from Krishnan *et al.* (2009).

shows the robustness of different axon groups to hyperpolarizing conditions is examined. In axons with greater I_H activity, there is greater inward rectification which allows the axons to better resist any hyperpolarizing conditions that are placed on them. Thus, in axons with greater inward rectification, the I/V curve will stay closer to baseline (closer to the y-axis), indicating greater excitability in these axons (less of a threshold increase). In Fig 2.4 above, it is important to note

that the axes are flipped so that the independent variable (conditioning strength) is plotted on the y-axis whereas the dependent variable (threshold reduction) is plotted on the x-axis. This is convention in AET literature.

Unlike TE, I/V reveals nothing about the temporal aspects of ionic currents, but allows for an examination of the rectification of I_H. The plot of I/V looks similar to conventional current-voltage plots, hence our abbreviation of current-threshold to I/V. Current-voltage plots from *in vitro* recordings look at how macroscopic conductances from single cells change across different voltages. I/V and its *in vitro* cousin both investigate the change in slope of the data curve as greater depolarizing or hyperpolarizing conditions are placed on the neuronal membrane. In other words, both I/V and current-voltage plots display the extent of rectification inherent in axon channels. Over the past 5 to 10 years, the I/V measure has illuminated differences in I_H between motor and sensory axons as well as between other axon populations (see Maurer *et al.* 2007, Jankelowitz *et al.* 2007, Krishnan *et al.* 2009, Trevillion *et al.* 2010, Howells *et al.* 2012, and Nodera and Rutkove 2012).

2.4.2.4 Recovery Cycle

The plot of recovery cycle (RC) reveals the oscillation in axon threshold which occurs after an action potential is fired, due to changes in multiple ionic conductances plus axon passive properties (i.e. myelin capacitance) which interact together (Fig 2.5). Therefore, the threshold oscillation measured in RC can be viewed as analogous to the oscillation in membrane potential which occurs after an action potential. In a nutshell, RC is obtained by varying the delay between a 1

ms-wide supramaximal pulse and a test pulse. Thus, the threshold change of an axon population is measured via the test pulse given at 18 different delays ranging from 200 to 2 ms after the supramaximal pulse. Three visible features of the RC plot are prominent: the relative refractory period (RRP), the superexcitability period, and the late subexcitability period. RRP is the delay period where excitability is decreased immediately after the supramaximal pulse. RRP generally ends (crosses the x-axis) at a delay of around 2.5 to 4 ms in RC. RRP is, unlike most other AET measures, highly dependent on body temperature. We did not use RRP for statistical comparisons in the "Results" chapter.

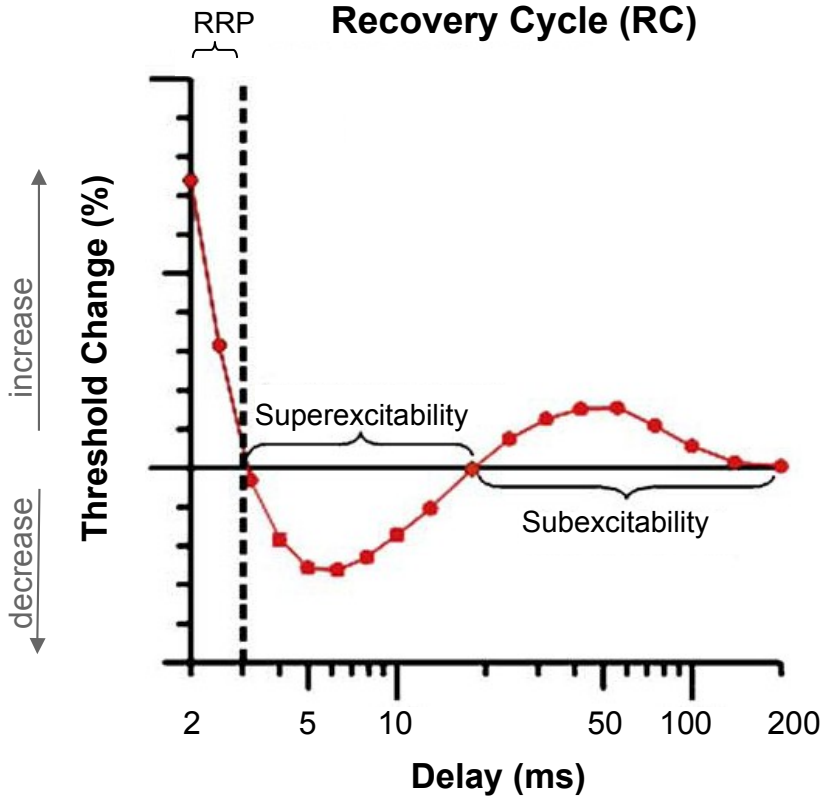


Figure 2.5. A representative RC plot. By investigating the oscillation in axon threshold which follows an action potential, RC can help assess the functioning of multiple ionic conductances along the axon proper, including Na^+ and slow K^+ conductances. Adapted from Krishnan *et al.* (2009).

Superexcitability is the period immediately after RRP when excitability is increased, due to a depolarizing afterpotential caused by capacitative discharge of the internode (Barrett and Barrett 1982, Kiernan *et al.* 2009). In many studies, superexcitability reaches a maximum at around 4 to 7 ms and for a few milliseconds afterwards superexcitability persists until the axon threshold goes back above baseline due to activation of slow K^+ conductance. Superexcitability begins at a delay as early as 2.5 ms after the supramaximal stimulus, and ends at delays of up to 25 ms. The change in this variable depends on the particular muscle nerve and species under investigation (George and Bostock 2007, Boerio *et al.* 2009, Mori *et al.* 2010, Burke *et al.* 2001). In general, all features of RC are affected by axon membrane potential. As previously stated, RC is also sensitive to the effects of temperature, with cooler temperatures causing a slightly reduced magnitude of superexcitability and a rightward shift of the RC oscillation waveform (Kiernan *et al.* 2001).

There are specific effects that membrane depolarization has on RRP and superexcitability. Depolarization acts to increase conductance through persistent Na^+ channels in the node, as well as causing increased inactivation of Na^+ channels, thereby increasing the duration of RRP. Also, depolarization of the axon membrane opens paranodal fast K^+ channels, which decreases the resistance of the internode and effectively decreases the magnitude of superexcitability. The major factor influencing late subexcitability is the activity of slow K^+ conductance, and with membrane depolarization the current through these outward rectifiers is increased as is the extent of subexcitability.

Hyperpolarization of the membrane generally has opposite effects to depolarization on the three main features of the RC waveform.

2.4.2.5 Rheobase and Strength-Duration Time Constant

Rheobase and SDTC reflect values which have not been influenced by a conditioning stimulus. These 2 AET measures are determined by five threshold currents having durations of 1.0 ms, 0.80 ms, 0.60 ms, 0.40 ms, and 0.20 ms. These values can be displayed in a strength-duration plot (Fig 2.6a), which relates threshold stimulus current (I) with stimulus duration (t), or alternatively a charge-duration plot (Fig 2.6b), which relates threshold stimulus charge (Q) with stimulus duration (t). The values of threshold stimulus charge corresponding to each of the pulse durations are fitted to Weiss's strength-duration equation, Q = a + b*t, in the form:

$$Q = Irh*(\tau_{SD} + t)$$

where Q is threshold stimulus charge, Irh is rheobasic current, τ_{SD} is SDTC, and t is pulse duration. Q is obtained by the product of I*t, where I is the threshold stimulus current and t is the stimulus duration. This equation assumes that threshold stimulus charge (Q) is directly proportional to stimulus duration (t), and a linear regression of Q on t determines Irh and SDTC (also denoted as τ_{SD} - Bostock *et al.* 1983). SDTC is calculated from the same physical measurements which are used to calculate rheobase, using Weiss's formula displayed in equation (1). When $t = -\tau_{SD}$ in this formula, then Q = 0. Thus, SDTC is the negative intercept in the linear relationship of Q and t (Fig 2.6b). We can see from the equation above that SDTC is actually the stimulus duration which corresponds to

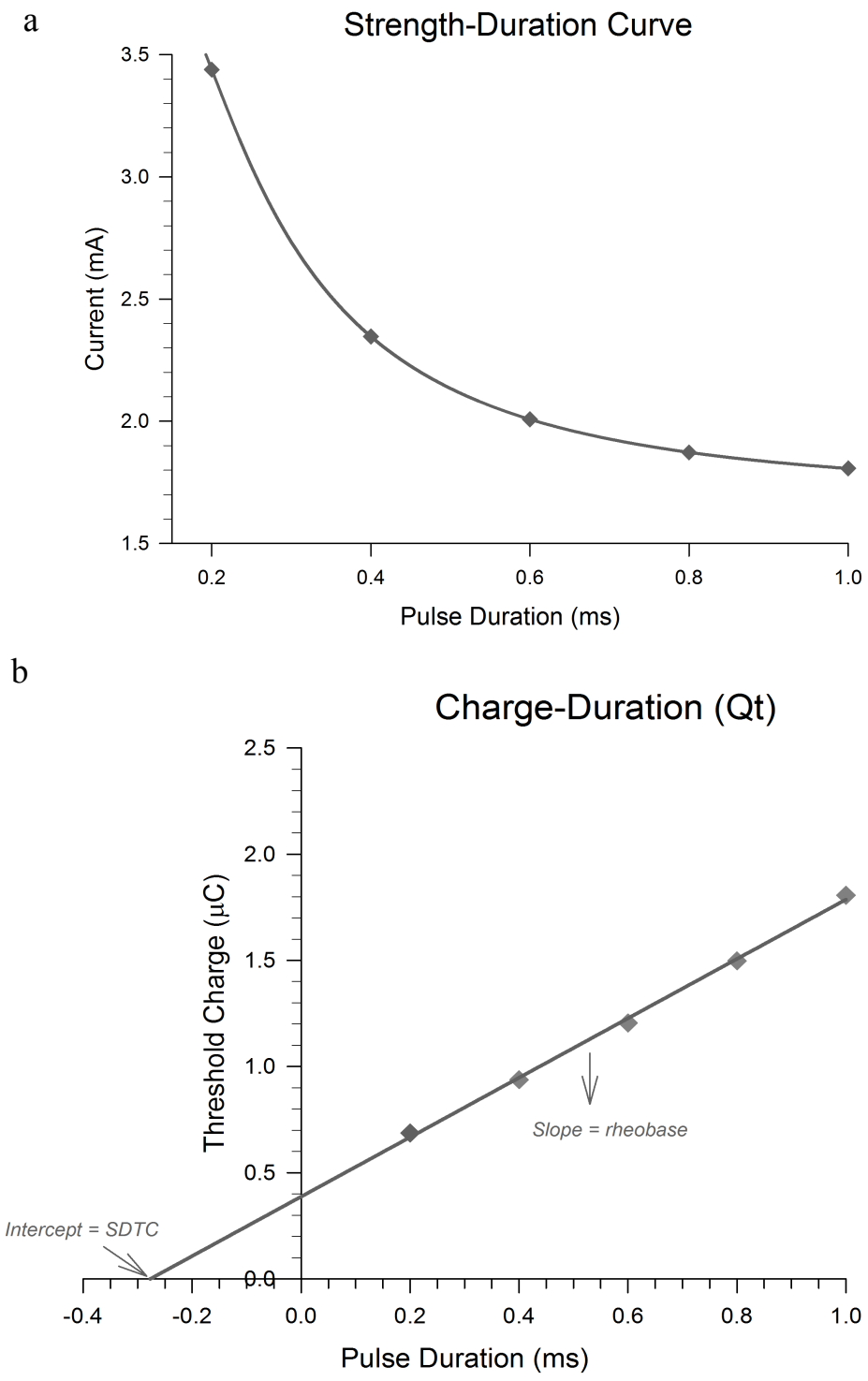


Figure 2.6. Unconditioned threshold measurements made at five different pulse durations resembles an exponential relationship between current amplitude and pulse duration as plotted in (a), or else a linear relationship between charge and pulse duration as plotted in (b). The charge-duration relationship provides values for rheobase and the strength-duration time constant (SDTC) through a linear regression of charge on stimulus duration. The equation in the text describes this linear relationship and shows that SDTC

is the x-intercept and rheobase is the slope of the line in (b). Fitting Weiss's strength-duration equation to the five data points (grey-filled diamonds) produces the regression equation Q = 0.39 + 1.40*t (grey line).

a threshold current that is exactly twice rheobase, since:

$$Q = I*t = Irh*(\tau_{SD} + t)$$

and when $t = \tau_{SD}$, $I = 2*Irh$

Therefore it is evident that SDTC (τ_{SD}) is equivalent to chronaxie, since chronaxie is defined as the stimulus current which is twice the rheobasic current. SDTC reflects the rate at which the threshold current changes as the test pulse duration gets larger. A larger SDTC means that threshold current decreases more slowly as the test pulse duration gets larger.

SDTC measures axon nodal properties and is influenced by factors such as temperature and the stimulus electrode geometry (Noble and Stein 1966, Bostock *et al.* 1983). Unlike rheobase, however, SDTC is relatively independent of the level of the target CMAP. A study of percutaneous stimulation in human single motor axons found that the SDTC of axons tracked at 30%, 60%, and 90% of the maximal CMAP had no statistically significant differences amongst themselves. However, this study also found the SDTCs of single motor axons, presumably having a lower-threshold than most other axons in the nerve, to be shorter than the SDTCs of a group of motor axons tracked at $CMAP_{30\%}$ (Mogyoros *et al.* 1996). SDTC is believed to correlate with Na^+ conductance, particularly persistent Na^+ conductance, such that larger SDTC values are associated with larger levels of persistent Na^+ conductance (Lin *et al.* 2002).

In our study, rheobase represents the theoretical minimum current - applied to a group of axons - required for an infinitely long stimulus pulse to elicit a

CMAP approximately 40% of maximal. Rheobase is numerically equivalent to the slope of the line which relates Q and t (Fig 2.6b). Importantly, rheobase is influenced by a variety of factors including the level of the target CMAP, temperature, and electrode placement. It has been advised that rheobase be used only for comparing two relatively similar preparations, which we have done in the present study, rather than used as an absolute value reflecting membrane properties (Mogyoros *et al.* 1996).

2.4.3 Spinal Cord Injury and Stroke – Models of Axon Inactivity?

Recently, AET has been used to study how axon bioelectric properties change after spinal cord injury (SCI) and stroke (Lin *et al.* 2007, Jankelowitz *et al*. 2007, Boland *et al.* 2009, Boland *et al.* 2011). Although the underlying causes contributing to the changes in peripheral axons following SCI and stroke remain unclear (due to extrasynaptic effects, altered MN metabolism, inactivity of axon changing membrane ionic conductances, or disruption of axon transport systems due to lesions, etc.?), serious abnormalities are evident in AET. This has contradicted conventional knowledge which espouses that peripheral motor axons are relatively unaffected after SCI. In a study seeking to ascertain the possible effects of inactivity on peripheral motor axons, 15 quadripalegic or paraplegic individuals, with time from injury ranging from 1 month to several years, had AET performed on the common peroneal (tibialis anterior) and median (abductor pollicus brevis) nerves (Lin *et al.* 2007). In the AET data, both median and peroneal axons exhibited similar changes: statistically significant smaller CMAPs, a shift to the right in the SR curve, a significant "fanning-in" of specific delays in

TE, a significant reduction in SDTC, and a trend towards a larger slope in hyperpolarized I/V. The severity of injury, determined by the ASIA Impairment Scale, was found to be uncorrelated with the changes to AET. In general, the findings from Lin *et al.* (2007) were rather similar to AET findings in depolarization-inducing ischaemia (Kiernan and Bostock 2000), generating the attractive hypothesis that a primary *peripheral* lesion was causing the observed changes to AET after SCI. The authors of Lin *et al.* (2007) argued that since median and peroneal axon changes were quite similar, however, a single peripheral lesion (due to something like bed rest, which would presumably only affect one of the two axon groups examined, or at least affect one more than the other) being the sole contributor to the observed axon changes was unlikely. Upon analyzing a sub-group of SCI patients with lower level thoracic lesions, it was found that peroneal axons exhibited greater abnormalities in AET than median axons. In this sub-group, peroneal axon changes were the same as the entire group except with a more prominent flattening of the RC curve (cf. Boland *et al.* 2011, discussed in the paragraph below). It was suggested that either a dysfunction in persistent Na^+ channels or a decreased conductance across all voltage-gated Na^+ channels could explain some of the results (such as the significant reduction observed in SDTC as well as the flattened RC curve in peroneal axons). Thus, although there was a tentative endorsement of two specific mechanisms contributing to axon pathophysiology after SCI, the general conclusion from Lin *et al.* (2007) was that a complex set of processes were responsible for the observed changes, and the changes were in turn thought to be

linked to decentralization and the consequent inactivity of peripheral axons.

Recently, two studies have longitudinally tracked the peripheral axon changes observed after SCI (Boland *et al.* 2009, Boland *et al.* 2011). The first of the two studies tracked changes in a single individual who suffered a hyperflexion-induced C6 fracture-dislocation due to a diving accident. AET was performed on peroneal axons innervating TA and on median axons innervating abductor pollicus brevis in this individual. Repeated AET measures were made on these two axon populations from 6 days after injury to the time the individual was discharged from the hospital (68 days after injury). At the earliest recording, AET of peroneal axons revealed a fanning-in of TE, steepening of the I/V curve, as well as a drastic flattening of the RC curve, in comparison with 95% confidence intervals from healthy controls. In contrast to the peroneal axons, AET on median axons did not reveal any significant abnormalities and values from the SCI individual did not stray outside of the 95% confidence intervals from healthy controls, though there was some similarity between peroneal and median axon AET data (i.e. there was some fanning-in of TE and a slight flattening of RC curve in the median axons). Over the 7 weeks from which AET was performed in the SCI patient, peroneal axons displayed sizable improvement and made recovery towards normative vales, although some parameters remained abnormal at day 68.

The above AET results from the single-case study have been supported in a more recent study (Boland *et al.* 2011) in which peripheral axon abnormalities were tracked longitudinally by performing AET on peroneal (tibialis anterior) and

median (abductor pollicus brevis) axons over 400 times in 11 individuals from the first day they were admitted until their discharge. The longest hospitalization period in any individual was 265 days. Like in the single-case study, AET revealed much more severe abnormalities in the peroneal axons compared to median axons. There were no statistically significant differences between median axons of SCI individuals and those of healthy controls in any of the AET measures. In the first day of admission to the hospital, individuals with SCI showed significant fanning-in of TE, steepening of the I/V curve, and flattening of the RC curve in peroneal axons (where there was an increased RRP plus decreased superexcitability as well as decreased subexcitability), again confirming the earlier findings from the single-case study. Also, as in the single-case study, peroneal axon AET data from the 11 individuals all displayed similar patterns of deterioration, which reached a peak abnormality at an average of 16.9 days after hospitalization. Recovery towards normative values typically occurred after 16.9 days after injury. Measures from conventional nerve conduction studies and EMG sampling did not significantly change throughout the AET recording period in the 11 patients, thus supplemental neurophysiological evidence supported the idea that AET findings were not caused by secondary abnormalities such as focal lesions due to bedrest pressures. In addition, the serum electrolyte levels of patients were within normal ranges throughout the recordings. The authors concluded that peripheral axons undergo serious functional changes during the acute phase of SCI, and that these changes occur in the absence of obvious systemic metabolic abnormalities. The onset of large changes in peroneal

axons coincided with the onset of hyperreflexia, and it was postulated that either (i) local metabolic changes at the MN soma, arising from possibly cord ischaemia or secondary metabolic/extrasynaptic effects, were partly responsible for these changes, and/or (ii) disruption of energy-dependent processes at the axon membrane, largely effecting the Na^+/K^+ pump, led to ischaemic-like conditions at the axon membrane. We must mention that the authors made no remark about the possibility of changes in I_H, which is known to directly affect TEh and I/V (Baker *et al.* 1987, Bostock and Baker 1988, Krishnan *et al.* 2009). TEh and hyperpolarizing I/V have shown significant differences in a large number of studies comparing AET of various axon groups (Kuwabara *et al.* 2000, Jankelowitz *et al.* 2007, Jankelowitz and Burke 2009, Murray *et al.* 2011, Howells *et al.* 2012, etc.). In fact, "TEh -40% (100-110 ms)" reveals the greatest number of significant differences in table 2.1, favoring the idea that I_H is a highly variable conductance in a wide variety of axons. Since TEh and hyperpolarizing I/V showed statistically significant differences between SCI patients and healthy controls in both of the longitudinal studies above (Boland *et al.* 2009, 2011), it is interesting that no commentary was provided in regard to the possibility of I_H plasticity in these two studies.

I_H has been implicated in a study which performed AET to investigate changes in median axons innervating abductor pollicus brevis in patients who had suffered a unilateral stroke and consequently were afflicted by unilateral hemiparesis (there were 4 individuals with pontine, 4 individuals with subcortical, and 4 individuals with cortical strokes – Jankelowitz *et al.* 2007). The patients in

this study all had predominantly motor symptoms with no sensory loss, and AET determined no change in the SR curve, SDTC or RC (Jankelowitz *et al*. 2007) in either the involved or uninvolved side. On the involved side, abnormalities were present solely in TEh and hyperpolarizing I/V: stroke patients had fanning-out of TEh and a less steep I/V curve in comparison with healthy controls (there were no significant differences found in these parameters between the uninvolved side of stroke patients and healthy controls). Utilization of a computer model of the axon led to the assertion that I_H in median axons is significantly reduced by 30% on the involved side of hemiparetic stroke patients, which the authors hypothesized was caused by inactivity of these axons (Jankelowitz *et al.* 2007). In addition to the changes in I_H, minimization of discrepancies between the axon model and experimental data in Jankelowitz *et al.* (2007) required that internodal leak conductance be reduced by 77%. These AET findings in stroke patients are opposite to those from the studies on SCI patients, where there was fanning-in of TE, steepening of the I/V curve, and an axon model suggested there was an increase in internodal leak conductance from 3.1 to 141 nS (Boland *et al.* 2009, 2011). Thus, although it is possible that the peripheral axon changes observed in SCI and stroke are partly due to alterations in their activity patterns, it is highly unlikely that inactivity is the main contributing factor, since if it was there should be greater similarity between the AET results in SCI and stroke. The underlying mechanisms of peripheral axon changes in both conditions remain mostly unknown, and it is clear that these two injuries lead to stark differences in axon pathophysiology as evidenced by AET. Studies which specifically examine the

effect of inactivity on peripheral AET measures could likely improve the interpretations of AET findings in SCI and stroke patients. For example, inactivity could be a shared factor affecting both pathologies or else it could be ruled out as having insignificant influence on AET in SCI and stroke patients.

2.4.4 Observed Axon Variation in Different Muscle Nerves and at Different Thresholds

To date, there have been 10 studies which have used AET to investigate possible differences between distinct healthy motor axon groups. These 10 studies are arranged chronologically as studies #1 through #10 in table 2.1: Kiernan *et al.* 1996 (study #1), Kuwabara *et al.* 2000 (study #2), Kuwabara *et al.* 2001 (study #3), Krishnan *et al.* 2004 (study #4), Bae *et al.* 2009 (study #5), Jankelowitz *et al.* 2009 (study #6), Mori *et al*. 2010 (study #7), Trevillion *et al.* 2010 (study #8), Murray *et al.* 2011 (study #9), and Nodera and Rutkove 2012 (study #10). The letters in the headings of each column represent a unique statistical comparison of two motor axon populations. All studies used repeated measuring of AET in separate motor axon populations *within* each subject, except comparison *n* which compared flexor carpi radialis axons in one set of individuals to abductor pollicus brevis axons in a different set of individuals from another study. Besides comparison *n*, all other comparisons distinguished between the two motor axon groups through (i) stimulating either the same nerve or two different nerves and tracking thresholds using CMAPs from two different muscles (found in comparisons *d, e, g, h, i, j, k, l, m, o,* and *v* in table 2.1), or (ii) stimulating the same nerve in two different locations along its length and then tracking thresholds using CMAPs from the same muscle (found in comparison *f*), or (iii) stimulating

Parameters	AET Results From Studies on Motor Axons											
	Study #1			Study #2			Study #3	Study #4			Study #5	
	a Median (APB) 30% vs. 70% CMAP	***b*** Median (APB) 30% vs. 50% CMAP	***c*** Median (APB) 50% vs. 70% CMAP	***d*** Median (APB) vs. Peroneal (EDB-a)	***e*** Median (APB) vs. Peroneal (EDB-k)	***f*** Peroneal (EDB-k) vs. Peroneal (EDB-a)	***g*** Median (APB) vs. Peroneal (EDB)	***h*** Peroneal (EDB) vs. (TA)	***i*** Tibial (AH) vs. Peroneal (TA)	***j*** Tibial (AH) vs. Peroneal (EDB)	***k*** Median (APB) vs. Ulnar (ADM)	***l*** Median (APB) vs. Ulnar (FDI)
TEd +40% (20-40 ms)				▲	NS	▲	▲				▲	▲
TEd +40% S2 accommodation				▲	▲	NS		□	□	□		
TEd +40% (100-110 ms)								▲	▲	▲	NS	NS
TEd +40% undershoot				▲	▲	NS		NS	NS	NS		
TEh -40% (20-40 ms)				□	NS	□		NS	NS	NS		
TEh -40% (100-110 ms)				□	NS	□	□*				NS	NS
TEh -40% overshoot				▲				NS	NS	NS		
Hyperpolarizing I/V				NS	NS	NS		NS	NS	NS		
Minimum I/V slope												
Hyperpolarizing I/V slope												
Relative refractory period (RRP)	NS	NS	NS	NS	NS	NS		□	□	NS	▲	NS
Superexcitability	NS	NS	NS	▲	NS	▲		▲	▲	NS	NS	NS
Subexcitability	NS	NS	NS	▲	▲	NS					▲	▲
Strength-duration time constant (SDTC)				NS	NS	NS					▲	NS

Parameters	AET Results From Studies on Motor Axons (cont'd)												
	Study #5 cont'd	Study #6		Study #7			Study #8			Study #9	Study #10		
	m Ulnar (FDI) vs. (ADM)	***n*** Median (APB-w) vs. (FCR)	***o*** Median (APB-e) vs. (FCR)	***p*** Rat tail 10% vs. 60% CMAP	***q*** Rat tail 10% vs. 40% CMAP	***r*** Rat tail 40% vs. 60% CMAP	***s*** Median 40% CMAP vs. single axons	***t*** Median 5% vs. 40% CMAP	***u*** Median 5% CMAP vs. single axons	***v*** Median (APB) vs. Ulnar (ADM)	***w*** Mouse tail 20% vs. 60% CMAP	***x*** Mouse tail 20% vs. 40% CMAP	***y*** Mouse tail 40% vs. 60% CMAP
TEd +40% (20-40 ms)	▲	▲	▲	▲	▲	NS		▲		NS			
TEd +40% S2 accommodation		▲	▲							NS			
TEd +40% (100-110 ms)	NS	▲		NS	▲	NS	▲	NS		NS			
TEd +40% undershoot		▲											
TEh -40% (20-40 ms)		□		▲	NS	NS				□	▲	▲	NS
TEh -40% (100-110 ms)	□	NS	▲	▲	▲	▲	□	▲		□	▲	▲	NS
TEh -40% overshoot		▲								▲			
Hyperpolarizing I/V										NS			
Minimum I/V slope		▲		NS	NS	NS	□		NS	NS			
Hyperpolarizing I/V slope		▲		▲	NS	▲							
Relative refractory period (RRP)	▲	□		NS	NS	NS				NS	NS	NS	NS
Superexcitability	NS	▲	▲	NS	NS	NS	NS	NS		NS	NS	NS	NS
Subexcitability		NS		NS	NS	NS	▲	NS	▲	NS	NS	NS	NS
Strength-duration time constant (SDTC)	▲	NS		▲	NS	NS	NS	NS		▲			

* The duration of the conditioning pulse in TE in study #3 was 300 ms while in all other studies it was 100 ms. Therefore, in study #3 where threshold change was measured at the end of the conditioning pulse, the parameter "TEh -40% (100-110 ms)" becomes "TEh -40% (300-310 ms)".

Table 2.1. The black triangles indicate where an AET parameter was found to have a significantly *larger* value for the axon population that is listed first in the axon pair of the column heading. The squares indicate a significant difference in the opposite direction. For example, the black triangle in the upper left-most corner indicates that ulnar axons innervating FDI have a significantly larger group mean in the parameter "TEd +40% (20-40ms)" in comparison with ulnar axons innervating ADM. NS refers to a nonsignificant result. A cell was left blank if no result was reported. The pattern of black triangles and squares in this table reflects the relationship *between* the 14 AET parameters included in this table. From this, we see that 8 of the parameters (pale green background) have a tendency to be interdependent with one another: "TEd +40% (20-40 ms)", "TEd +40% (100-110 ms)", "TEd +40% undershoot", "TEh -40% overshoot", "Hyperpolarizing I/V slope", Superexcitability", "Subexcitability", and "SDTC". It is possible that there is co-variation amongst these 8 parameters. Note that studies 7 and 10 dealt with AET measures from rodents, while all of the other studies involved *in vivo* measurements from human axons. Also, to maintain harmony between the figures and text in this thesis, for measurements made at the end of the 100 ms conditioning current in both TEd and TEh, we have indicated the delay as "100-110ms", since the conditioning current in TE does not begin until x_{delay} = 10 ms. In the literature this delay is denoted instead as "90-100 ms". Unless otherwise noted, the target CMAP for threshold tracking was wherever the SR curve was steepest, which is usually around 40% of a maximal CMAP.

the same nerve and measuring from the same muscle but tracking thresholds using different CMAP amplitudes.

The methods used in all AET paramaters assume that the axons being tested remain identical as long as the target CMAP remains constant throughout the recording. Therefore, when comparing AET parameters tracked at different target CMAPs within the same nerve, it is assumed that different groups of motor axons are being compared. This was done in comparisons *a, b, c, p, q, r, s, t, u, w, x,* and *y* in table 2.1. In total, there were 25 comparisons made in the 10 studies, with each comparison analyzing two motor axon groups. The statistical analyses included paired samples t-tests, Wilcoxon's signed rank tests, Student's t-tests, and Mann-Whitney's U-tests. Of these 25 comparisons, 24 were unique. The single redundancy is found in studies 2 and 3 where median axons (CMAP measured at APB) were compared to peroneal axons (CMAP measured at EDB). The

abbreviations for the muscles in table 2.1 are as follows: ADM stands for abductor digiti minimi, AH for abductor hallucis, APB for abductor pollicus brevis, EDB for extensor digitorum brevis, FCR for flexor carpi radialis, and FDI for first digitorum interosseus. To some of the muscles in the column headings we added a hyphen and a single letter, since these investigated AET parameters at specific electrical stimulation sites along the nerve. Therefore, "-a", "-w", and "-k" indicate stimulation sites at the ankle, wrist, and knee, respectively. We will now discuss the trends seen in these 10 studies.

First, it must be explained that the ordering within each pair of motor axon groups, shown in the column headings of table 2.1, imparts meaning to the significant differences. The significant differences are indicated by the black triangles and the squares. As an example, in comparison *d* we have written "Median (APB) vs Peroneal (EDB-a)" in the column heading. Each triangle found under this column indicates that median axons innervating APB gave a statistically significant *greater* value than peroneal axons innervating EDB, for any given parameter/row a triangle is located in. A square also indicates that a significant difference was found but in the opposite direction. Therefore, the first square under the column "Median (APB) vs Peroneal (EDB-a)", found in the fifth row from the top, is indicating that median axons innervating APB had a significantly *lower* value for the parameter "TEh -40% (20-40 ms)" when compared to peroneal axons innervating EDB.

Table 2.1 shows that for 20 out of the 25 comparisons, there has been a statistically significant difference found in at least 2 of the 14 AET parameters.

In fact, 12 of the 25 comparisons revealed significant differences in 4 or more AET parameters. It is interesting to note that in 7 out of 9 complete comparisons of different motor axons innervating the same muscle[1] there were significant differences in at least 2 out of the 14 excitability parameters (in comparisons *p, q, r, s, t, w, and x*). This provides some evidence that there are distinct "types" of axons within a single muscle nerve, although none of the studies from table 2.1 have related AET measurements to other properties such as MN somatic bioelectric properties or motor unit mechanical properties. Therefore, the implications of these axon differences are unclear.

That axons innervating the same muscle can have statistically significant differences in AET parameters likely came as a surprise to the authors of the earlier studies in table 2.1. The differences found between axons of the same nerve, as well as axons innervating the same muscle, were originally interpreted as arising from stimulation site-dependent differences in axons (see Kuwabara *et al.* 2000, Kuwabara *et al.* 2001, Krishnan *et al.* 2004, for examples). This was perhaps because the early studies were aiming to investigate excitability along the length of an axon through stimulating different sites of the same nerve while tracking CMAPs from different muscles (therefore, in addition to comparing stimulation site-dependent differences amongst axons, these studies were also comparing motor axons which were anatomically and functionally-distinct). In addition, the first study to investigate differences amongst motor axons of different thresholds found no significant differences in the RC parameters (Kiernan *et al.* 1996; however, compare this with Trevillion *et al*. 2010 in which

significant differences were found in subexcitability between axons tracked at different thresholds). Later, however, it was conceded that differences due to axon stimulation site and/or axon length are insufficient to explain the growing list of significant differences between distinct motor axon groups (Kuwabara 2009, Jankelowitz and Burke 2009). The more recent studies have focused on differences in AET between motor axons of the same muscle yet different thresholds (Mori *et al.* 2010, Trevillion *et al*. 2010) or between axons of different nerves (Murray and Jankelowitz 2011) and have interpreted these differences as variations amongst motor axon sub-groups rather than stimulation site-dependent variations. The most obvious trend from these studies, then, is seen not as much in the results as in the interpretations over the years. It has been a fairly recent endeavour to intentionally investigate variations in motor axon bioelectric properties, and it is now recognized that axon function and activity patterns have some part to play in these variations (Burke 2007, Kuwabara 2009).

2.4.4.1 Possible Interdependence of AET Parameters

In addition to imparting meaning to the black squares and triangles in table 2.1, the ordering of each pair in the column headings has been arranged in such a way as to show the most coherent trend possible. In the table we see that 8 out of 14 AET parameters display only one type of symbol (only triangles) in their row. These parameters are "TEd +40% (20-40 ms)", "TEd +40% (100-110 ms)", "TEd +40% undershoot", "TEh -40% overshoot", "Hyperpolarizing I/V slope", "Superexcitability", "Subexcitability", and "SDTC". Let us label these 8 AET parameters as "group A parameters" in the present discussion involving the 10

studies shown in table 2.1. The pattern seen in group A parameters provides evidence that there is interdependence (i.e. co-variation) amongst these parameters, above and beyond the expected interdependence due to shared conditioning or shared properties. Therefore, certain combinations of parameters such as "TEd +40% (20-40 ms)", "TEd +40% S2 accommodation" and "TEd +40% (100-110 ms)" are expected *a priori* to have a larger interdependence with each other than with other parameters because they share the same conditioning stimulus and are all known to be influenced by slow K^+ conductances. No matter how table 2.1 is arranged, however, the significant differences found in these 3 parameters do not produce a pattern of only triangles or only squares, and this contrasts with the striking pattern seen with the 8 group A parameters[2]. In terms of probability, if the 8 group A parameters in table 2.1 were completely independent of one another, the likelihood of pure chance producing the pattern of significant differences seen in the table is 1 in 32,000[3]. In addition, if the group A parameters had just slight interdependence (insomuch as shared experimental factors or redundant physiological properties allowed), it would be unlikely that these 8 parameters would reveal 42 significant differences that all perfectly match with one another. Because of this evidence for interdependence amongst the group A excitability parameters, this predicts interdependence between the physiological processes/properties underlying those parameters (these physiological processes/properties are discussed below in section 2.4.6.2). The 10 studies in table 2.1 thus provide novel evidence of interdependence amongst motor axon bioelectric properties.

There is one lone parameter, "Hyperpolarizing I/V", which did not have a significant difference reported in any of the 25 comparisons from the 10 studies. The other remaining 5 AET parameters in table 2.1 have a mix of both symbols in their row (both triangles and squares) and therefore the directions of the significant differences are not consistent in these parameters. Let us call these 5 parameters "group B parameters". These parameters are "TEd +40% S2 accommodation", "TEh -40% (20-40 ms)", "TEh -40% (100-110 ms)", "Minimum I/V slope", and "RRP". Although some interdependence is expected between the physiological properties/processes underlying the group B parameters (due to the sharing of physiology between some of these parameters, discussed above)[4], group B parameters are different from group A parameters in that a repeating pattern of symbols does not exist. Thus findings from group B parameters may support the view that *multiple* physiological processes/properties contribute to the significant differences found between distinct groups of motor axons in table 2.1 (since if all of the significant differences in the 14 parameters were due to a single underlying property, we should expect that each and every parameter would show only one type of symbol in their row). In addition, AET literature assumes that a mix of active properties (ionic conductances) plus morphological/passive properties influence each and every AET parameter. Also, some axon morphological/passive properties are believed to co-vary with each other (Rushton 1951, Jack 1975) so it is possible that group A parameters have a larger influence from co-varying passive axon properties (*i.e.* myelin conductance, axon membrane resistance, axial resistance, etc.) than the group B

parameters do. On the other hand, group A parameters are known to be influenced by active properties (see below) and therefore co-variation of active properties underlying the group A parameters is also possible. It should be re-iterated that at present there are no studies which have examined the extent to which active motor axon properties co-vary, such as how the density of nodal Na^+ channels might co-vary with the density of internodal slow K^+ channels. Since there has been no detailed investigations dealing with the implications of variation in motor axon active properties (as opposed to the great number of studies which have investigated the implications of variations in MN somatic and motor unit properties), and since it is obvious from table 2.1 that there are variations in motor axon properties, this paucity of knowledge provides compelling reasons for more detailed investigations of how motor axon properties vary across different groups (i.e. across different muscle nerves, different motor unit types, etc.). The present study, although not an exhaustive investigation, intends to shed more light on the variation in motor axon AET parameters across different axon groups.

2.4.4.2 Underlying Physiological Properties

Overall, the 10 studies from table 2.1 strongly suggest that multiple physiological processes/properties are responsible for the significant differences found between motor axon groups. And, given that AET literature has demonstrated that active properties influence all of the AET parameters (involving Na^+ conductances, slow K^+ conductance, I_H, and probably fast K^+ conductance as well. See Bostock *et al*. 1998 and Krishnan *et al.* 2009 for reviews. Also, see Baker *et al.* 1987, Bostock and Baker 1988, McIntyre *et al.* 2002, Trevillion *et al.* 2007, and Jankelowitz *et*

al. 2007 for direct experimental or modeling evidence for the influence of active properties in AET), it is quite likely that multiple *active* properties contribute to the significant differences in table 2.1, although morphological/passive properties are probably also playing a role. With regard to active properties, significant differences in "TEd +40% (20-40 ms)", "TEd +40% S2 accommodation", "TEd +40% (100-110 ms)", and "TEd +40% undershoot" are very likely being partly caused by differences in slow K^+ conductance (see Baker *et al.* 1987, Bostock and Baker 1988, Schwarz *et al.* 2006), while significant differences in TEh -40% (20-40 ms)", "TEh -40% (100-110 ms), "TEh -40% overshoot", "Minimum I/V slope" and "Hyperpolarizing I/V slope" are very likely being caused, in part, by I_H (see Baker *et al.* 1987, Bostock and Baker 1988, Bostock *et al.* 1998, Howells *et al.* 2012). Where significant differences are found for the parameter RRP in table 2.1, differences in Na^+ channel kinetics (inactivation) are partly responsible for this (see Hodgkin and Huxley 1952, Burke *et al.* 1998, McCintyre 2002, Krishan *et al*. 2009). The interplay of properties responsible for significant differences in Superexcitability is in some ways more complicated. It is usually assumed in AET studies that the parameter "Superexcitability" reflects the internodal capacitative discharge of the axon within a few milliseconds following an action potential. This capacitative discharge has effects on axon membrane potential (Barrett and Barrett 1982, Bostock *et al*. 1998, Krishnan *et al.* 2009). A modeling study (McIntyre *et al.* 2002), however, has found that both morphological/passive properties (axon diameter and internodal capacitance) as well as active axon properties (Na^+ channel activation and inactivation time constants as well as fast

K^+ conductance) may influence superexcitability. Therefore, it is possible that superexcitability is influenced by a complex mix of multiple active and multiple passive properties, and it is interesting to recall that from table 2.1 there is the suggestion that superexcitability co-varies with the other parameters of group A. The third and final parameter of RC, "Subexcitability", is thought to be influenced by membrane potential but is also greatly influenced by the slow K^+ conductance (see Baker *et al.* 1987, Lin *et al.* 2000, Krishnan *et al.* 2009). Lastly, significant differences in the parameter "SDTC" are partly caused by differences in density, distribution, or kinetics of persistent Na^+ channels (see Bostock and Rothwell 1997, Bostock *et al.* 1998).

In regard to the differences between motor axons with different thresholds (comparisons *p, q, r, s, t, u, w, x,* and *y*), there are progressive changes in some excitability parameters as axon electrical recruitment threshold gets larger. A consistent change was seen in "TEd +40% (20-40 ms)", "TEh -40% (20-40 ms)" and "TEh -40% (100-110 ms)". In the comparisons which detected a statistically significant difference in at least 1 of these 3 parameters (comparisons *p, q, r, s, t, w,* and *x)*, the lower threshold axons were found to have greater excitability increases in "TEd +40% (20-40 ms)" and/or less excitability decreases in the two TEh parameters (in other words, there was a fanning-out in TEd and a fanning-in of TEh for the lower threshold axons). These TEh findings point towards lower threshold axons having greater I_H than higher-threshold axons. The idea that lower threshold axons have greater activity of I_H than higher threshold axons has been tested and supported by a recent study (Trevillion *et al*. 2010). Furthermore,

the fact that there was noticeably fewer significant results found in the I/V parameters than in the TEh parameters suggests a difference in the kinetics of I_H between lower threshold and higher threshold axons (data from hyperpolarizing I/V and hyperpolarizing TE normally co-vary because they are both affected by I_H). These parameters, Hyperpolarizing I/V and the two TEh parameters, have indeed recently been utilized to test the hypothesis that differences in I_H contribute to the differences between motor and sensory axons (Howells *et al.* 2012, Nodera and Rutkove 2012). It is possible that since "Hyperpolarizing I/V" examines threshold changes at a longer delay than in "TEh -40% (100-110 ms)", at 200 ms rather than at 100-110 ms, the longer period of activity of I_H in I/V provides greater opportunity for the threshold change in higher-threshold axons to be "clamped" and brought closer to the threshold change in lower-threshold axons, thereby obscuring differences which are seen in "TEh -40% (100-110 ms)". In other words, higher-threshold axons may have more of the slower-activating I_H (and/or less of the faster-activating $I_{H,}$ as proposed by Nodera and Rutkove 2012) than lower threshold axons, but this difference in kinetics is obscured after I_H has been activated for long durations such as in hyperpolarized I/V. As previously mentioned, I_H has been found to differ between motor and sensory axons (Nodera and Rutkove 2012, Krarup and Moldovan 2012, Howells *et al.* 2012) as well as to change after stroke (Jankelowitz *et al.* 2007 – see section 2.4.3). In the 10 studies depicted in table 2.1, "TEh -40% (100-110 ms)" gave the most number of significant differences, thirteen out of twenty five comparisons, and as stated above "TEh -40% (100-110 ms)" is known to be influenced by I_H.

Therefore, I_H appears to be an important part of how axons ionic conductances vary according to motor unit type.

Also, in regard to motor axons with different thresholds, it is interesting that the parameters "TEd +40% (20-40 ms)" and "TEh -40% (100-110 ms)" appear to be interdependent with each other. From table 2.1 we see the trend of lower threshold motor axons having higher excitability in both "TEd +40% (20-40 ms)" and "TEh -40% (100-110 ms)" when compared to the higher threshold axons[5]. "TEh -40% (100-110 ms)" is influenced by underlying active axon properties (*i.e.* I_H), but what about "TEd +40% (20-40 ms)"? As discussed above, this parameter is influenced by passive axon properties as well as by slow K^+ currents (see Bostock and Baker 1988, Bostock *et al.* 1998, Trevillion *et al.* 2007). "TEd +40% (20-40 ms)" may also be influenced indirectly by fast K^+ currents through internodal resistance changes caused by opening/closing of fast K^+ channels during the prolonged subthreshold depolarization in this parameter (Trevillion *et al.* 2007). It is possible that there is interdependence between "TEd +40% (20-40 ms)" and "TEh -40% (100-110 ms)" in axons of different thresholds and, therefore, this promotes the possibility of interdependence of specific active axon properties. However, it is possible that "TEd +40% (20-40 ms)" may be influenced by I_H active at resting membrane potential and therefore the apparent interdependence between these two AET parameters may be due simply to shared physiology. If experiments were to be done that can more fully elucidate the relationships among AET parameters across different motor axon groups, the possibility that functionally distinct "types" of motor axons will emerge seems

intriguing. And, if motor axons can indeed be classified into functionally distinct "types", one wonders if the classification of these groups would match the classification of their motor units and muscle fibers. For example, do axons of fast fatigable motor units arrange into a distinct group, while axons of fast fatigue-resistant arrange into another distinct group?

2.5 Summary

A multitude of studies have demonstrated that intrinsic MN somatic properties, muscle properties, and axon CV can adapt to different activity patterns. There is observable fine-tuned coordination amongst and between MN somatic and muscle properties in both normal conditions and after activity is learned, in multiple limb muscles and in multiple species (Burke *et al.* 1973, Gustaffson and Pinter 1984, Zengel *et al* 1985, Munson *et al.* 1986, Cope *et al.* 1986, Gardiner and Kernell 1990, Gordon *et al.* 1997, Munson *et al.* 1997, Gossen *et al.* 2003). The current understanding of MN plasticity is limited by the fact that important motor axon electrophysiological properties may also adapt along with their MN soma and motor unit properties, although technical obstacles have prevented a rigorous examination of many axon properties. AET is a relatively new method which provides a way around these technical obstacles by using axon threshold to investigate axon bioelectric properties *in vivo*. From AET studies, it is apparent that there are multiple axon bioelectric properties which vary according to the muscle innervated as well as according to the axon threshold level (see table 2.1 and associated text). Importantly, the idea has recently been introduced that AET

parameters. This highlights the question of whether axon variations and/or adaptions are coordinated with MN somatic and muscle unit properties. It is possible that motor axon bioelectric properties are coordinated in a way that reveals distinct "types" of motor axons.

Distinct "types" of motor axons would recall the *heuristic* appeal of Burke's three main physiological motor unit types. However, in section 2.2.1 we discussed Erlanger and Gasser's observations that some notable axon bioelectric properties vary continuously, such as CV and action potential size, which ostensibly supports the idea there are *not* functionally-distinct axon types. It must be emphasized that it is quite possible that all of the AET parameters vary continuously. Despite this continuous variation, axons may still be classified into certain "types" since there is a large pool of AET parameters which can be drawn from in order to optimize the separation between axon "types". This separation of axons into distinct "types" would be similar to the way that motor units typically arrange into Burke's three generalizable types of fast fatigable, fast fatigue-resistant, and slow. Though it is recognized that many motor unit and MN properties vary continuously, the motor units themselves can still usually be classified into Burke's three main motor unit "types". In Fig 2.7, adapted from Burke *et al.* (1973), it can be seen that four motor unit properties, two or three of which exhibit continuous variation, can distinguish three motor unit "types" in cat medial gastrocnemius. When "tetanic tension", "twitch contraction time", and "fatigue index" are plotted together along with the description of whether there was any "sag", the data can be shown to separate motor units into Burke's three

Properties that vary continuously can help reveal distinct "types"

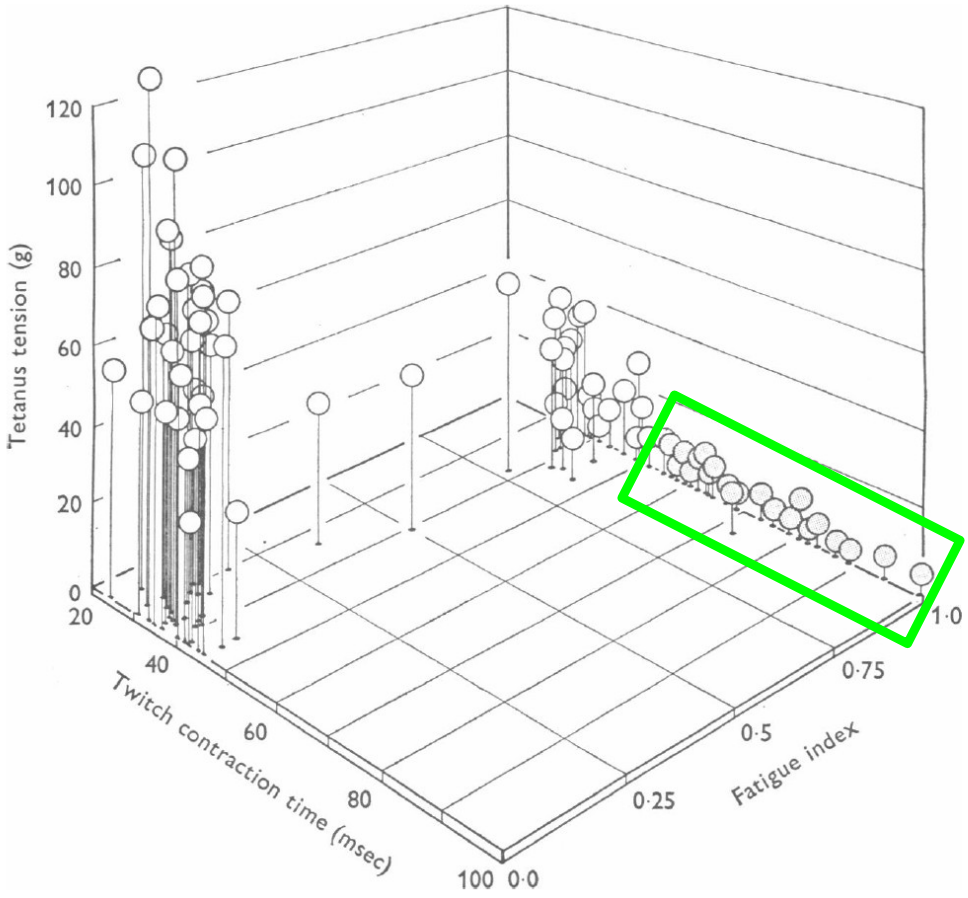


Figure 2.7. Motor unit mechanical properties were recorded in about 100 motor units from cat medial gastrocnemius in the study by Burke *et al.* (1973). This 3d plot displays the "tetanic tension", "twitch contraction time", and "fatigue index" of each of these motor units. The presence or absence of a fourth property, "sag", is indicated by the green rectangle placed over the motor units which showed an absence of sag. All other motor units outside of the green rectangle showed a presence of sag. Arguably, three out of four of these properties vary continuously. By considering all four properties together, a pedagogical purpose can be seen in distinguishing between three distinct "types" of motor units in cat medial gastrocnemius. From Burke *et al.* (1973).

motor axon variations are due to adaptations to different activity patterns (Burke 2007, Kuwabara 2009), although there has been very little discussion about the functional implications of this for the MN or motor unit. Patterns evident in table 2.1 demonstrate the possibility of interdependence amongst some motor axon

main “types”. Thus, the heuristic appeal of classifying motor units into distinct “types” is supported by using continuously varying properties (Fig 2.7).

Taken together, the findings from the AET studies favor the idea that motor axon variations occur mainly via differences in voltage-gated ion channels (see Cormery *et al.* 2005 and Gardiner 2006 where this is postulated to occur at the MN soma. Also, see Rannou *et al.* 2009 for evidence of significant differences in Na^+ conductances across muscle fibers with different myosin heavy chain isoforms). Variations in morphological or passive axon properties are possibly also contributing to these axon variations. If the kinetics/distributions/densities of multiple axon ion channels coordinate with each other, it would seem likely that this coordination would affect the entire MN and that it would be functionally relevant for motor units. It is known that axon CV correlates with Burke's motor unit types (*i.e.* fast fatigable motor units very often have a higher CV than slow motor units), but do other active axon properties correlate with MN and muscle properties? Also, we know from Zengel *et al. (*1985) that somatic bioelectric properties can augment overall motor unit function (i.e. a high somatic input resistance and low rheobase MN typically innervates fibers that are more fatigue-resistant and have lower contractile forces, therefore this matching of properties enhances the ability of the motor unit to sustain activity over long periods of time), but could variations in motor axon ionic conductances also augment the function of motor units? AET has provided a great opportunity to investigate these questions in the present study.

Notes

1. Kiernan *et al.* 1996, shown first in table 2.1, also investigated differences between axons innervating the same muscle but of different thresholds. This study, however, restricted its investigation to only RC and so only dealt with three AET parameters: "RRP", "Superexcitability", and "Late Subexcitability". Furthermore, this investigation was not specifically aiming to investigate the differences between motor axons but rather was seeking to elucidate the differences in recovery cycle between motor and sensory axons. In addition, this study did not provide information on the type of statistical tests performed and the lack of significant findings is in contrast to the significant differences found in closely related comparisons *s* and *u* from table 2.1.

2. From table 2.1 we see that wherever a significant difference between two motor axon groups was detected in table 2.1, each of the 8 excitability parameters in group A produces a perfectly consistent direction of difference (i.e. only one type of symbol in their row). No matter how table 2.1 is re-arranged (by flipping the order of certain columns/comparisons and thereby changing the pattern of symbols in each row), this same striking uniformity cannot be created for any combination of a larger set of parameters. Although this does *not* mean there is statistically significant co-variation amongst the parameters or the physiological properties underlying group A parameters, table 2.1 nonetheless reveals that there is a definite trend for the direction of significant differences in the group A parameters to match together. This provides indirect evidence that some of the underlying physiological properties may co-vary.

3. If we were to assume that the 8 group A parameters were completely independent, then wherever a significant difference (SD) is found in table 2.1 we would expect a random occurrence of two possibilities (for the independence assumption to be fully met we must also assume that each of the comparisons/columns of axons were independent, which is supported by the fact that each comparison contains a unique axon pair except for comparison *g*. Also, every column except for two, *h* and *i*, have a unique set of SDs, providing more support for the assumption of independence between the comparisons/columns).

The two possibilities are that either the first axon group in each pair has a statistically significant greater value or it has a statistically significant smaller value than the other group in that pair. Thus, an analogy can be made to the 42 significant differences found across 18 separate comparisons for the 8 group A parameters. The analogy can in fact be made to the tossing of a coin in 18 separate series of tosses, with each series consisting of a variable number of tosses ranging from 1 toss to 6 tosses. In total, across all 18 series, there will be 42 coin tosses. Also, to make the analogy more accurate, before the coin tossing begins let's say that Professor X decides that the outcome of the first toss in each series will be excluded plus an additional 9 tosses will be excluded at random across the 18 series (these excluded tosses represent the degrees of freedom across the 18 comparisons having an SD in the group A parameters in table 2.1). Student Y then performs the 42 coin tosses. After the exclusion of the first toss in each of 18 series as well as the random exclusion of 9 coin tosses, a total of 15 coin tosses are counted by Professor X. To their astonishment, Professor X and Student Y find that 'heads' turned up in all of these 15 coin tosses. This finding is similar to that which has occurred for the 8 group A parameters of table 2.1. What is the probability of this happening purely by chance? For the 8 group A parameters, the probability that the pattern of SDs arose by pure chance can be calculated as:

$$2 \text{ ^- } (n - df)$$

where 2 represents the two possibilities for a SD, n is the number of significant differences found for the 8 group A parameters in table 2.1, and df is the degrees of freedom. The number of significant differences is 42. The degrees of freedom is 27 (the number of rows plus the number of columns contained in the sub-table of significant differences for the group A parameters). Therefore, the probability is:

$$2^{-(42-27)} = 2^{-15} = 1 / 32768 = 0.00003$$

From this we can state that it is unlikely that the 8 group A parameters are completely independent. However, we cannot rule out that the apparent interdependence of SDs is due to all of the parameters sharing the same underlying physiology. But since the AET literature has demonstrated that various underlying properties are unique to certain parameters in group A, shared physiology is highly unlikely to be the the sole contributing factor here. It is likely that co-variation of multiple physiological properties underlying the group A parameters has contributed to the interdependence of the SDs. One caveat is that interdependence amongst the comparisons/columns may have increased the likelihood of interdependence between the SDs, which could then be mistakenly attributed to interdependence of the group A parameters. This influence must have been small, though, since 17 of the 18 comparisons had a unique set of SDs and also since all of the comparisons which shared similar axons all produced a unique set of SDs. In addition, interdependence between the comparisons/columns seems less likely when considering that the pattern of SDs in some closely related or identical comparisons (i.e. comparisons *k* and *v*, which

are identical comparisons with axons from the same muscle nerves and the same thresholds but they only share the same SD pattern in 1 out of the 7 parameters which contained a SD for these comparisons) were not more similar to each other than the pattern of SDs in unrelated axons (i.e. comparisons *p* and *m*, which have axons from different muscle nerves in different species and they share the same SD pattern in 2 out of the 6 parameters*)*.

4. There is some degree of interdependence which can be shown in other sub-groups of the 14 AET parameters (due to shared physiology and perhaps co-variation of physiological properties/processes as well), and table 2.1 can be rearranged to show evidence of interdependence amongst other combinations of parameters. However, since these other combinations consist of fewer significant differences and fewer parameters, there is a greater likelihood that shared physiology was a strong contributing factor in these other instances of interdependence (i.e. parameters which are strongly influenced by slow K^+ current should be expected to show some degree of interdependence in their significant differences). Obviously, it goes without saying that if the significant differences *in all parameters* were to appear to be perfectly interdependent (i.e. if there were only triangles or only squares in all 14 rows), this would endorse the possibility that a shared mechanism (i.e. a single property) was responsible for all of the significant differences found in table 2.1. Since this was not the case, and also since we discuss in section 2.4.6.2 that AET literature has established multiple active axon properties which influence the parameter set, the trend of significant differences in group A parameters is likely due to true interdependence amongst axon properties and not due purely to chance. Furthermore, the group A parameters are shown in the literature to be influenced by different active axon properties, and this provides evidence that the interdependence seen in these parameters is not due to shared physiology alone.

5. Although comparison *s* from table 2.1 does not reveal a significant difference at "TEd +40% (20-40 ms)", this comparison nevertheless did reveal a significant difference in "TEd +40% (100-110 ms)" whereby axons of single units (presumably lowest-threshold axons) actually had *smaller* excitability increases than axons tracked at higher target levels. This is contradictory from the pattern seen for "TEd +40% (20-40 ms)", and this may suggest that "TEd +40% (20-40 ms)" and "TEd +40% (100-110 ms)" have separate and independent underlying physiological properties. This is unlikely, however, for three reasons: (i) because of the evidence that these parameters, both part of the group A parameter set discussed in note 1 and in section 2.4.6.2, had significant differences which seemed to be interdependent, (ii) similar passive/morphological axon properties likely affect these two parameters, and (iii) there is likely only a single, shared active axon property which influences both of these parameters - slow K^+ conductance.

Therefore, the unique pattern seen in comparison *s* from table 2.1 may indicate that axons from single units are not on the same "continuum" of progressive

changes in certain excitability parameters as the higher threshold axons are. In other words, there may be an abrupt change in some properties of the lowest-threshold axons (*i.e.*"TEd +40% (100-110 ms)"), which is in contrast to the progressive and orderly changes seen when moving from higher to lower threshold axons (when moving from CMAPs of 60% to 40% to 10% to 5% of maximal, we see that "TEd +40% (20-40 ms)" gets progressively larger, but this trend does not appear extend to the lowest-threshold single axons). The recruitment patterns of electrically stimulated axons is obviously of great interest here.

Chapter 3

Methods

3.1 Experiments and Recordings

Fig 3.1 depicts a schematic of the experimental set-up. A total of 14 female Sprague-Dawley rats, weighing 280 ± 50 g (mean ± SD), were used as experimental models in this study. A weight of 280 g in female Sprague-Dawley rats corresponds to an age of approximately 90 days, which represents young but sexually mature females (cf. Yang *et al.* 2000, George and Bostock 2007). Threshold tracking was used to ascertain 5 main AET measures: TE, I/V, RC, rheobase, and SDTC. These measurements were performed separately on axons innervating TA and axons innervating SOL in the right and left hindlegs, so that each rat had the 5 AET measures determined in both axon groups in each leg. For the purposes of this thesis, we used data from right legs only. The "TA axon group" refers to axons within the motor nerve innervating TA while the "SOL axon group" will refer to axons within the motor nerve innervating SOL. Controlled stimuli were delivered using QTRAC software (©Institute of

Neurology, Queen Square, London, UK) using a isolated bipolar constant current stimulator (Digitimer DS5, Digitimer Ltd., Welwyn Garden City, Hert., UK). Experimental recordings generally took 30 minutes to complete in each specimen, although in 5 rats the recordings took longer because some AET measures had to be reassessed. In these cases where an AET measure was repeated, the CMAP noise grew unacceptably high - usually during RC or TE - and so the electrodes were repositioned and the necessary test was repeated.

All experimental recordings were completed within 3 hours of administering the first dose of anaesthetic. In 9 rats, anaesthesia was administered via intra-peritoneal injection of a mixed dose of 60 mg/Kg ketamine and 7.2 mg/Kg xylazine. Five rats were anaesthetised with an intra-peritoneal injection of 60 mg/Kg sodium pentobarbital. Data from these rats were only used for the results described in section 4.2. The five rats anaesthetised with sodium pentobarbital were not used in statistical comparisons of TA and SOL axons. After the initial dose of sodium pentobarbital, the animals were given intra-peritoneal injections (1/3 of initial dose) when a corneal or leg-withdrawal reflex was observed. While still anaesthetized, each animal was euthanized by cervical dislocation after experimentation

3.1.1 Signal Processing

CMAPs were sampled at a rate of 10 KHz and were amplified, filtered (10-1000 Hz) and digitized by an 8-bit A/D board (National Instruments DAQ-6062e, Austin Texas, USA). Noise introduced from nearby power sources was removed in real time using a Hum Bug 50/60 Hz Noise Eliminator (Quest Scientific

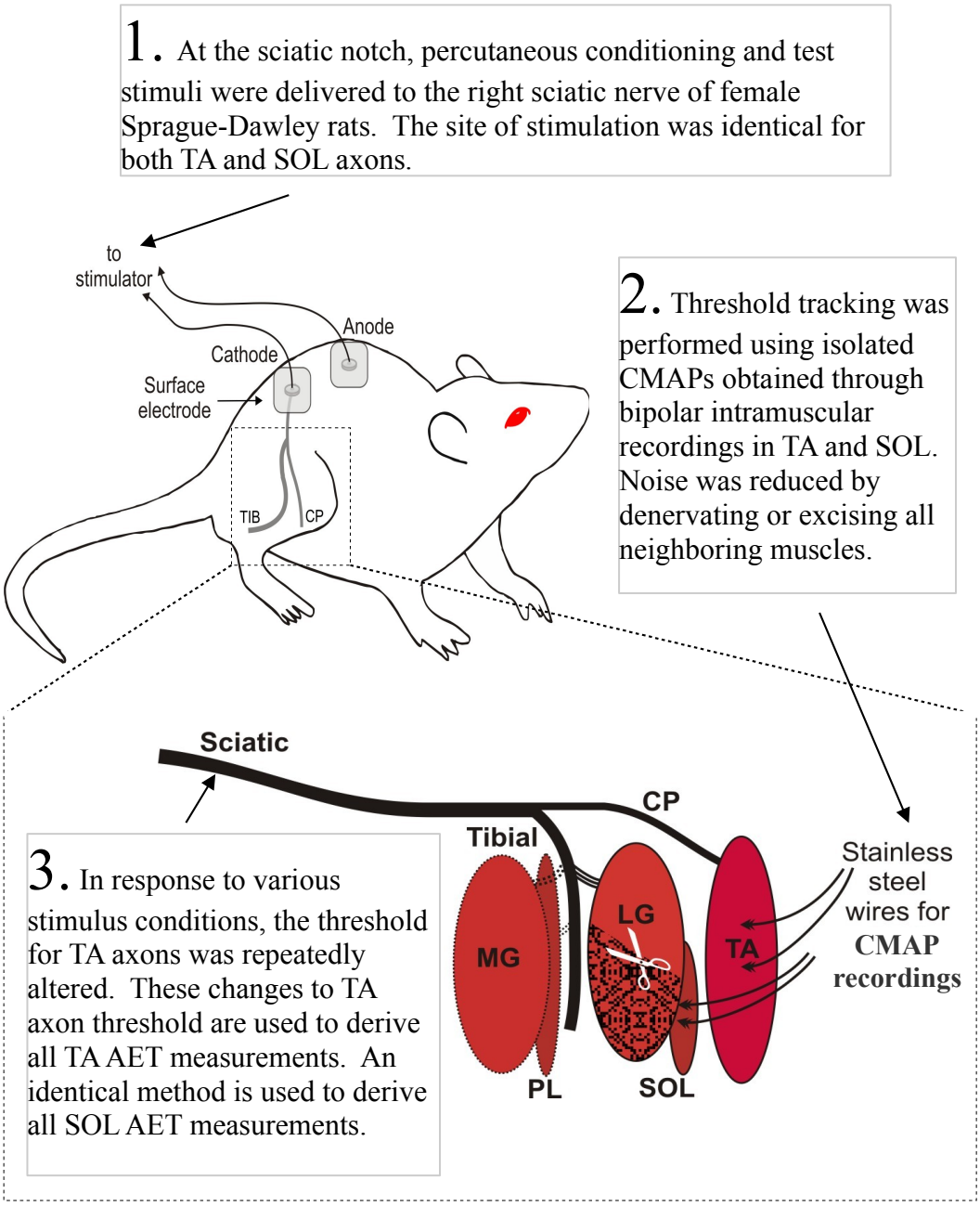


Figure 3.1. Overview of the experimental setup. Electrical stimulation of the sciatic nerve elicited CMAPs in both TA and SOL. Threshold tracking of a "target CMAP" was used as feedback control of the test threshold current amplitude. Recordings took approximately 15 min for each axon group in each rat. AET measures are derived from the changes in axon threshold recorded throughout threshold tracking.

Instruments, North Vancouver, BC, Canada). In cases of excessive levels of noise, we also implemented a 60 Hz notch filter in 6 rats to help remove noise originating from grid power.

The target CMAP window is monitored automatically through repeated delivery of an unconditioned test pulse every time an advance is made to a new delay, new conditioning strength, or new pulse duration. The recording time between each advance varies depending on the AET measure, preparation stability, and tracking parameters. Generally, the length of time for a single AET measurement ranges from approximately 2 to 10 seconds. The purpose of this continual monitoring is to check for any drift in the target CMAP and the accompanying changes in excitability of the preparation. By using the ±7.5% window of error for the target CMAP, the excitability of the preparation can change slightly without greatly affecting the measurements or the quality of threshold tracking. If physiological drifts in target CMAP were not buffered in this way, any time a drift occurred there would be an erroneous impression of poor tracking. For example, several responses falling just short of the target would seem to indicate that threshold tracking is performing poorly, and so an adjustment in tracking methods would be required, although often a slight physiological shift in excitability may be the true cause of this. Therefore, an adjustment in the tracking parameters often provides little-to-no benefit.

The maximal CMAP was monitored at the start and at the end of recording in both TA and SOL AET, in order to detect any large drifts in the target CMAP. In addition, since every AET measurement is heavily dependent on muscle

responses, the waveforms of CMAPs were carefully monitored in each animal experiment in order to detect changes in shape or distortion due to noise. If large changes in waveform shape were observed, such as a ringing or a multi-phasic oscillation (most often seen during RC), then the relevant data was discarded in that rat and we then repeated data collection. This occurred in 5 rats.

3.1.2 Electrical Stimulation and Recordings

A schematic of the experimental setup is shown in Fig 3.1. Percutaneous electrical stimulation was delivered via Ag/AgCl 3M Red Dot electrodes, with the active electrode placed over the sciatic notch and the reference electrode placed over the lumbar vertabrae at the midline of the back, for both TA and SOL axons. To record a bipolar intramuscular CMAP signal in TA, a hypodermic needle (26G) was first inserted near the motor endplate on the proximal 1/3 of the muscle, and a second hypodermic needle was placed about 2.5 mm apart from the first. Prior to insertion into the muscle, teflon-coated stainless steel monofilament wire (Cooner Wire 765-40; 40G) was then threaded through each of the hypodermic needles, with approximately 3 mm of teflon removed from the end of the wire to enable CMAP recordings. The wires extended from the tip of the hypodermic needle by ~2 mm and were bent to form a hook. Insertion of the hypodermic needles (27G) in SOL was similar to TA except they were placed about 5 mm apart because of the orientation of the motor endplate and muscle fibers in SOL. After the stainless steel recording electrodes were securely placed in each muscle, the needles were removed.

In order to minimize cross-talk from other muscles and thus reduce CMAP

noise for threshold tracking, extensor digitorum longus, flexor digitorum longus, medial gastrocnemius and plantaris were all denervated, while approximately the distal 3/5 of lateral gastrocnemius (LG) was excised. LG could not be denervated because SOL axons are intertwined with LG axons under a shared epineurium. Also, LG could not be completely excised since SOL axons pass through the LG muscle. A rectal thermometer monitored internal body temperature, which was maintained between 34.5°C and 38.5°C by a heating lamp. After all experimental recordings were made, each of the AET measurements which would later be subjected to statistical analyses were first screened for outliers by investigating data points greater than 1.5x interquartile ranges away from the group mean (we screened for outliers in 10 different TA axon TE delays plus 10 different SOL axon TE delays, and in 16 TA axon RC delays plus 16 SOL axon RC delays, etc.; in total, there were 52 TA AET measurements and 52 SOL AET measurements which we used in our statistical analyses). There were a couple outliers in some of the AET measurements made on TA and SOL axons, although no consistent pattern was observed (i.e. a large number of outliers in TA axon measurements coming from a single rat) and therefore there was no need to remove any outliers from our analysis.

3.2 Data Analyses

For 3 of the 5 main AET measures (TE, I/V, and RC), we used univariate repeated measures analyses (also commonly known as mixed between-within ANOVAs) to compare TA and SOL axons. Independent samples t-tests were used in rheobase

and SDTC, as well as for the comparisons of the anaesthetics in section 4.2. For comparisons between the two axon groups which involved three or more similar measurements, we used a common modification of repeated measures analysis called the Greenhouse-Geisser estimate, since "time" was a within-subjects factor and it is often known to violate the sphericity assumption. Bonferoni post-hoc examinations were performed after any significant interaction effect was found in a repeated measures analysis. In statistical comparisons utilizing independent samples t-tests (in the comparisons of rheobase and SDTC from section 4.1.6 and also in the comparisons between anaesthetics in section 4.2), we used a modified t-test provided by SPSS whenever Levene's test revealed a violation of the assumption of equal variances. In addition, where multiple t-tests were performed on a similar physiological measure, which occurred only in section 4.2, we performed a Bonferoni correction using the formula, $\beta = \alpha/m$, in order to reduce Type I statistical errors. Other studies using AET have suggested that particular parts (measurements) within the five main measures in AET are of greater interest. Our analysis focused on these parts, as they likely represent crucial points where axon bioelectric properties may diverge.

3.2.1 Assumptions of Data Analyses

For the purposes of this thesis we analyzed data from right legs only, although we collected TA and SOL AET data from both the left and right hindlegs[1]. For both repeated measures analyses and independent samples t-tests, multiple assumptions of the dataset must be met before proceeding with the statistical tests. These assumptions include a continuous scale for dependent variable measurements,

random sampling, independence (for independent samples t-tests only), normality, and equal variances (also known as the sphericity assumption in univariate repeated measures analyses). Generally, if these assumptions are not met and no appropriate adjustments are made, the T or F statistic and the associated p-value will be invalid.

First, we can safely state that the dependent variable was measured on a continuous scale and that our samples (rats) were taken at random. Second, in each of the independent samples t-tests, we tested for homogeneity of variance using Levene's test. In the rare case where the assumption of equal variance was violated, we used a modified t-test provided by SPSS in which no assumption of equal variance is needed. Essentially, all that is changed in these t-tests is a reduced degrees of freedom. This lowers Type I statistical errors. In addition, the sphericity assumption was not met in a number of repeated measures analyses. This requires an explanation as to why repeated measures were kept as a method of comparison. If an appropriate correction factor is employed when an assumption such as sphericity is not met, p-values can be adjusted to validate the statistical test at hand. In our analysis, we encountered violations to the assumption of sphericity in our within-subjects factor "time". But Tabachnik & Fidell (2007) state that when "time" is used as a within-subjects factor in repeated measures, the sphericity assumption is routinely violated because "things measured closer in time tend to be more highly correlated than things measured further away in time". We therefore followed convention by using a modified repeated measures analysis with adjusted degrees of freedom and an adjusted F

statistic (as well as an adjusted p-value) through the Greenhouse-Geisser method, in all of our repeated measures analyses. This type of repeated measures is robust to violations of sphericity and significantly reduces Type I error rate due to sphericity (Tabachnik & Fidell, 2007), therefore the violation of sphericity here can be safely ignored.

Normality was objectively assessed by the Shapiro-Wilk test and it was found that a small number of groups of data (groups which are separated by AET measure plus the factors "time" or "conditioning strength"*)* violated the normality assumption. Specifically, 2 out of 20 data groups in TE and 1 out of 30 data groups in I/V were not normally distributed (all 18 groups in I/V Slope, all 32 groups in RC, the 2 SDTC groups and 2 rheobase groups were found to be normally distributed; n = 9 for all data groups except for those in RC where n=7) when an individual significance level of $\alpha = 0.05$ was used. Such a small number of normality violations in TE and I/V provides little impetus for a data transformation. Although there are normally no adjustments used for multiple normality tests (i.e. something akin to a Bonferoni adjustment), it is possible that the 3 normality violations actually do not meet a "familywise" significance level, since there was such a large number of individual tests of normality. In addition, our small sample sizes may have influenced sensitivity to normality tests. Nevertheless, since 101 out of a total of 104 data groups were found to be normally distributed by the Shapiro-Wilk test, this provides evidence that the validity of our statistical conclusions are not compromised by a comparatively low number of normality violations.

Lastly, in our comparison of ketamine/xylazine vs. sodium pentobarbital, we used independent samples t-tests. Since ketamine/xylazine was administered in a group of rats separate from the rats given sodium pentobarbital, then AET measurements from the ketamine/xylazine rats do not depend on AET measurements from the sodium pentobarbital rats.

Notes

1. Although we analyzed data from right legs only, we used paired samples t-tests to compare right vs. left TA axons as well as right vs. left SOL axons in the 52 AET measurements. Out of 104 measurements (52 AET measurements in TA axons plus 52 measurements in SOL axons), we found 3 AET measurements which showed significant differences between the left and right sides. These differences were restricted to TE in SOL axons. Specifically, the significant differences between right and left SOL axons were found in: TEd +40% at a delay of 20-30 ms ($T_{12} = 2.232$, $p = 0.045$), TEd +40% at a delay of 120-150 ms ($T_{12} = -2.977$, $p = 0.012$), and in TEh -40% at a delay of 120-150 ms ($T_{12} = 2.495$, $p = 0.028$). However, if a post-hoc Bonferoni correction is applied in the form:

$$\beta = \alpha/m$$

where β is the adjusted p-value, α is the familywise significance level (0.05), and m is the number of individual t-tests performed within each main AET measure, *we see that only one significant difference remains at a familywise significance level of 0.05, in TEd +40% at a delay of 20-30 ms* (in TEd +40% there are 3 measurements made at three different delay groups, so that $\beta = 0.05/3 = 0.017$). A significant difference in only 1 out of 104 measurements, which just reaches familywise significance, likely reflects a statistical Type I error.

In contrast, by using repeated measures as our main statistical analysis when comparing TA and SOL axons, we eliminated the need to perform multiple t-tests and therefore decreased the risk of Type I errors in comparisons of TA vs. SOL axons.

Chapter 4

Results

4.1 Comparison of TA and SOL Axons

4.1.1 A Note on Values Plotted and Reported in this Chapter

All data corresponding to boxplots was reported in the text as mean ± SEM. For data corresponding to other plots, values were reported in the text the same way they were plotted, as mean ± 95% confidence interval (CI). For all comparisons, an alpha level of 0.05 was chosen for rejecting the null hypothesis (this includes analyses with multiple comparisons, where a familywise alpha level of 0.05 was chosen). For all boxplots, the mean is represented by the dashed line, while the lower and upper edges of the box represent the 1st and 3rd quartiles of the data sample, respectively. The lower and upper whiskers represent the 5th and 95th percentiles of the data sample, respectively.

4.1.2 Stimulus-Response Curve

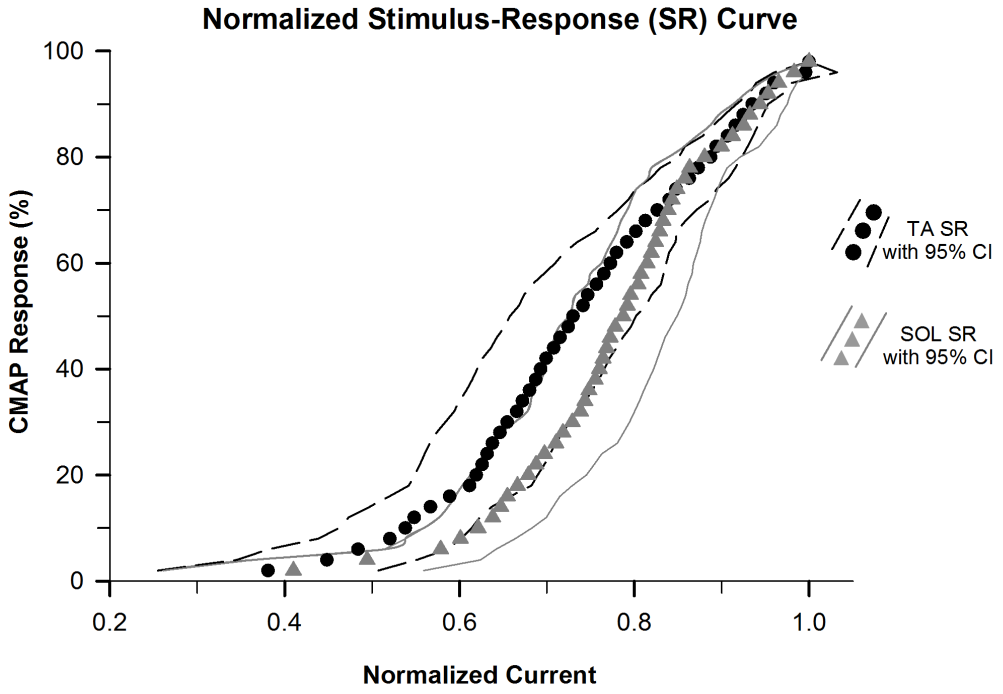


Figure 4.1. The average of the normalized SR curves for TA axons (n=9) and SOL axons (n=9). SR curves are used to determine the 5 main AET measures.

Figure 4.1 displays the TA and SOL axon SR curves, showing that TA axons tend to have a higher proportion of their axons recruited at lower normalized currents than SOL axons. SR was not subjected to any statistical analysis because it is not intended to measure any specific aspect of axon physiology, and a difference in the SR curve does not implicate any specific differences in axon physiology. The SR curve is obtained at the beginning of the experimental session in each subject, as a necessary measurement which is used to determine the 5 main AET measures later on during the experimental protocol.

4.1.3 Threshold Electrotonus

Figure 4.2 displays the response of TA and SOL axons to the 100 ms conditioning pulse applied within the 200 ms recording window of threshold electrotonus (TE). The conditioning pulse onset occurs at x_{delay}=10 ms and pulse offset at x_{delay}=110 ms. As can be expected, axon threshold changes are greatest immediately after the pulse onset and offset, and this is due mainly to a passive spread of charge to the axon node (termed the "F phase" for the pulse onset at 10 ms). It is important to note that threshold change, measured in percentage, is measured at ~26 time points throughout the 200 ms recording interval, although only about 10 of these time points were used in planned statistical comparisons. These statistical comparisons are performed using pre-selected groups of time points (henceforth referred to as "delay groups"): 20-30 ms, 100-109 ms, and 120-150 ms for both the TEd +40% and the TEh -40% tests. Statistical comparisons in the hyperpolarizing tests, TEh -40% and TEh -20%, included only the 100-109 ms and 120-150 ms delay groups. These three delay groups that were chosen for statistical tests are shown in the literature to be key points where axon properties commonly diverge (see Schwarz *et al.* 2006, Bae *et al.* 2009, Isose *et al.* 2010, for examples). In other words, these delay groups represent a crucial interval of time where a conductance or multiple conductances has/have a large influence on threshold. An example is slowly activating K^+ conductance, which oppose depolarization and cause threshold to increase during the 20-30 ms interval in TEd +40% and TEd +20%). For the statistical analyses in each of the four TE tests, threshold values from multiple discrete time points were averaged together

within each delay group, to keep the sample size at n = 9 for both TA axons and SOL axons.

For TEd +40%, the conditioning pulse causes depolarization of the motor nerve and a decreased threshold for the duration of the conditioning pulse and a very brief period (*i.e.* less than 1 ms for both TA and SOL axons) following its offset. For TEh -40%, the conditioning pulse causes hyperpolarization of the nerve and an increase in threshold for the duration of the conditioning pulse and for a moderately brief period (*i.e.* less than 20 ms for both TA and SOL axons) following its offset. Although quantitative differences between the TA and SOL axons are of primary importance, describing the qualitative differences between these two axon groups will be useful for initially orientating the reader. From Fig 4.2 it can be seen that in TEh -40%, where the hyperpolarizing conditioning pulse is being applied, there is an obvious divergence between the TA and SOL axon responses. SOL axons have greater excitability (i.e. less of a threshold increase) than TA axons across these hyperpolarized TE measurements. A second difference exists between TA and SOL axons after the pulse offset in TEh -40%, and about 40 ms after the pulse offset (*i.e.* where delay is between 110 and 150 ms) SOL axons have a larger excitability than TA axons. In addition, after the pulse offset in TEh -40%, it is apparent that SOL axon excitability goes above baseline before TA axons.

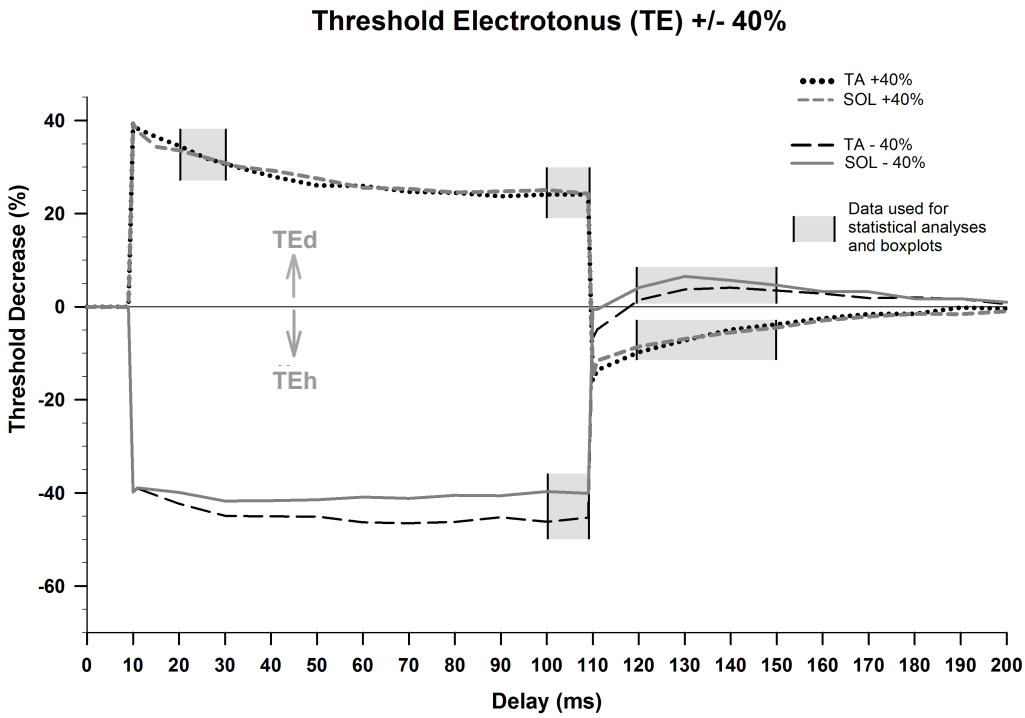


Figure 4.2. Threshold current changes in response to a conditioning pulse were recorded at discrete delays over a period of 200 ms in each TE test, although planned statistical comparisons of TA and SOL axons were done at three specific "delay groups" within the TE tests. Values at each of the delays plotted here are an average from 9 rats. Note that a decrease in threshold current is plotted above the y-axis, while an increase in threshold current is plotted below it, following convention in AET literature. The delay groups are represented by light grey boxes bounded by vertical black borders, on a layer behind the data. This figure displays two TE tests, TEd +40% and TEh -40% (For the sake of clarity, we have excluded the other two TE tests from this figure, which are qualitatively very similar and which reveal similar quantitative differences between TA and SOL axons). Differences between TA and SOL axons can be seen in TEh -40%.

Although these two observations reveal visual differences between the TA and SOL axon responses to TE, it is premature to think of these differences as being physiologically relevant. The results below dealing with TE will examine whether or not there are statistically significant differences between TA and SOL axons in subsets of TE data that were selected a priori. It is possible that multiple statistically significant main effects can arise out of the TE repeated measures

analyses, although the question still remains whether or not these are originating from multiple physiological processes (i.e. multiple axon ionic conductances causing multiple significant differences) or if they are the result of a single process (i.e. a single ionic conductance causing multiple differences). A large portion of the Discussion chapter will be dedicated to an examination of the various differences between TA and SOL axons, and which motor axon physiological properties may be functionally related to which differences in the AET measures.

4.1.3.1 Threshold Electrotonus Repeated Measures Analyses

Quantitative differences between TE of TA and SOL axons were investigated using repeated measures analyses. There were 4 repeated measures analyses performed in total. For TEd +40% and TEd +20%, all three delay groups (20-30 ms, 100-109 ms and 120-150 ms) were included in each repeated measures analysis. TEh -40% and TEh -20% had the 20-30 ms delay group excluded and therefore only two delay groups (100-109 ms and 120-150 ms) were included in each repeated measures analysis. Note that the corrected degrees of freedom and resulting p-values given below are determined by the Greenhouse-Geisser method, which is robust to violations of the sphericity assumption and which is the most conservative repeated measures test (Tabachnik & Fidell 2007).

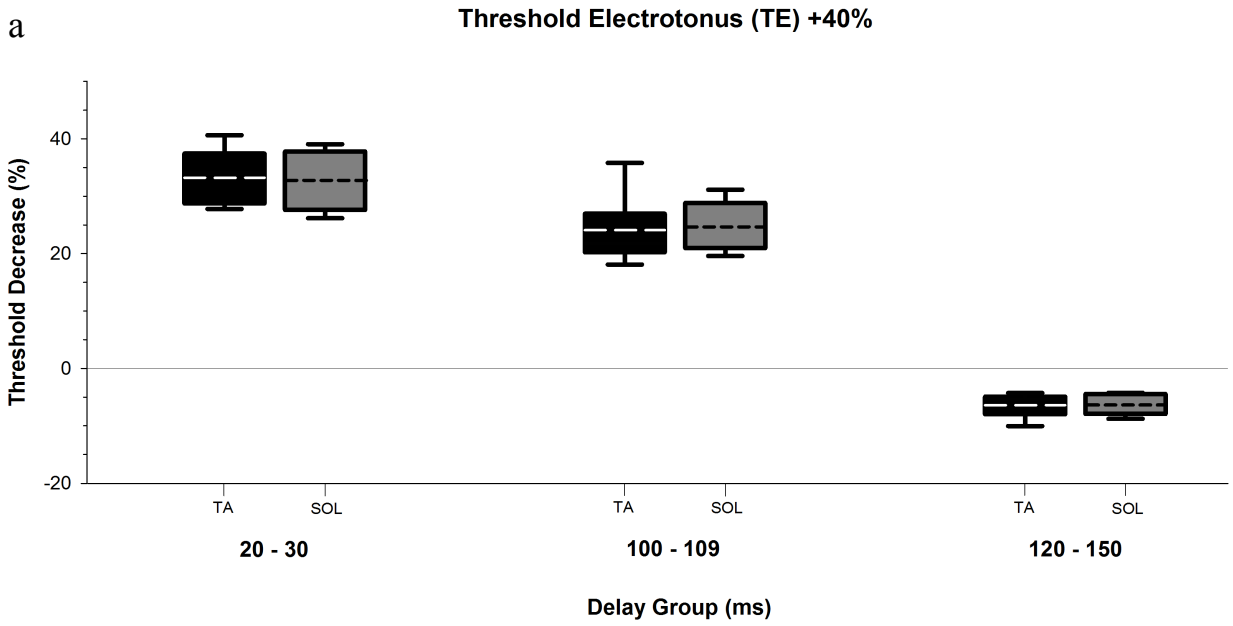
TEd +40% (Fig 4.3a): In this repeated measures comparison, there was no significant main effect of axon group ($F_{1, 8} = 0.005$, $p = 0.95$) nor a significant interaction effect between the two within-subjects factors, axon group and delay group ($F_{1.75, 14.27} = 0.41$, $p = 0.65$). For the 20-30 ms delay group, mean TA axon

threshold decrease was 33.22 ± 1.61% while mean SOL axon threshold decrease was 32.79 ± 1.69%. For the 100-109 ms delay group, mean TA axon threshold decrease was 24.11 ± 1.81% while mean SOL axon threshold decrease was 24.70 ± 1.42%. For the 120-150 ms delay group, mean TA axon threshold decrease was -6.40 ± 0.65% while mean SOL axon threshold decrease was -6.35 ± 0.59%.

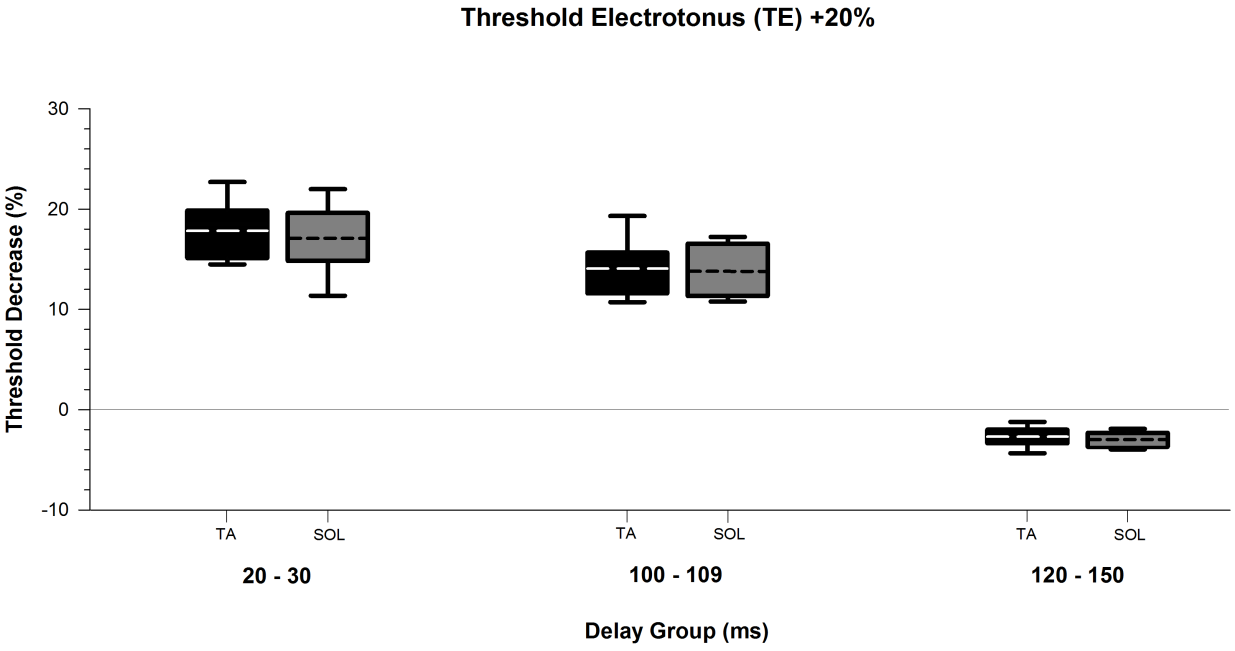
TEd +20% (Fig 4.3b): There was no significant main effect of axon group ($F_{1, 8} = 0.31$, $p = 0.59$) nor a significant interaction effect ($F_{1.38, 11.00} = 0.29$, $p = 0.67$). For the 20-30 ms delay group, mean TA axon threshold decrease was 17.84 ± 0.95% while mean SOL axon threshold decrease was 17.09 ± 1.07%. For the 100-109 ms delay group, mean TA axon threshold decrease was 14.08 ± 0.92% while mean SOL axon threshold decrease was 13.81 ± 0.92%. For the 120-150 ms delay group, mean TA axon threshold decrease was -2.70 ± 0.32% while mean SOL axon threshold decrease was -2.99 ± 0.25%.

TEh -40% (Fig 4.3c): There was a significant main effect of axon group ($F_{1, 8} = 16.78$, $p = 0.003$). However, there was also a significant interaction effect as well ($F_{1, 8} = 6.21$, $p = 0.037$ - see Fig 4.3c) and Bonferoni post-hoc comparisons determined that TA axon threshold decrease was significantly smaller (had a larger negative value) than SOL axon threshold decrease at 100-109 ms ($T_{26} = -4.76$, $p < 0.001$). For the 100-109 ms delay group, mean TA axon threshold decrease was -45.77 ± 3.02% while mean SOL axon threshold decrease was -39.90 ± 2.83%. For the 120-150 ms delay group, mean TA axon threshold decrease was 3.20 ± 0.70% while mean SOL axon threshold decrease was 5.23 ± 0.76%.

a


Threshold Electrotonus (TE) +40%
Threshold Decrease (%)
40
20
0
-20
TA
SOL
TA
SOL
TA
SOL
20 - 30
100 - 109
120 - 150
Delay Group (ms)

b


Threshold Electrotonus (TE) +20%
Threshold Decrease (%)
30
20
10
0
-10
TA
SOL
TA
SOL
TA
SOL
20 - 30
100 - 109
120 - 150
Delay Group (ms)

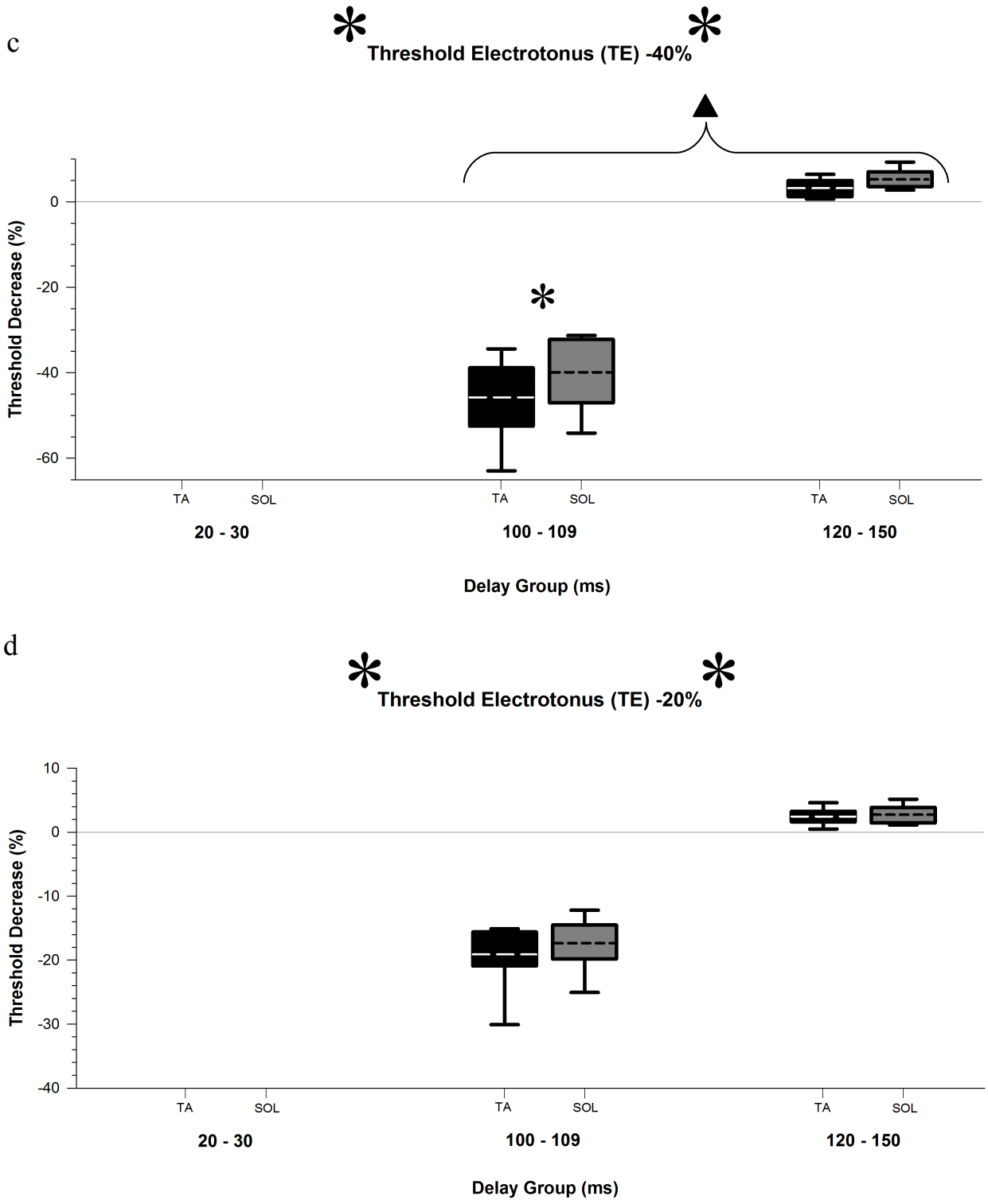


Figure 4.3. Comparisons at specific delay groups within the TE tests reveal significant differences between TA and SOL in hyperpolarizing TE tests. For each of the four TE tests, a repeated measures test (a mixed between-within ANOVA) was performed in which there was one between-subjects factor (rat) and two within-subjects factors (axon

group and delay group). A significant main effect of axon group was found for both TEh -40% and TEh -20% (see text for details). This is indicated by the large asterisks (*) on the left and right sides of each of the titles for these tests. A significant interaction effect was found in TEh -40%, and is shown by the black-filled triangle (▲). Post-hoc comparisons determined that the 100-109 ms delay group had significant differences between TA and SOL axons in TEh -40%, and this is shown by a small asterisk (*) above the corresponding pair of boxes.

TEh -20% (Fig 4.3d): There was a significant main effect of axon group ($F_{1, 8} = 6.49$, $p = 0.034$) and there was no significant interaction effect found ($F_{1, 8} = 2.04$, $p = 0.19$). For the 100-109 ms delay group, mean TA axon threshold decrease was -19.10 ± 1.56% while mean SOL axon threshold decrease was -17.37 ± 1.29%. For the 120-150 ms delay group, mean TA axon threshold decrease was 2.44 ± 0.40% while mean SOL axon threshold decrease was 2.75 ± 0.47%.

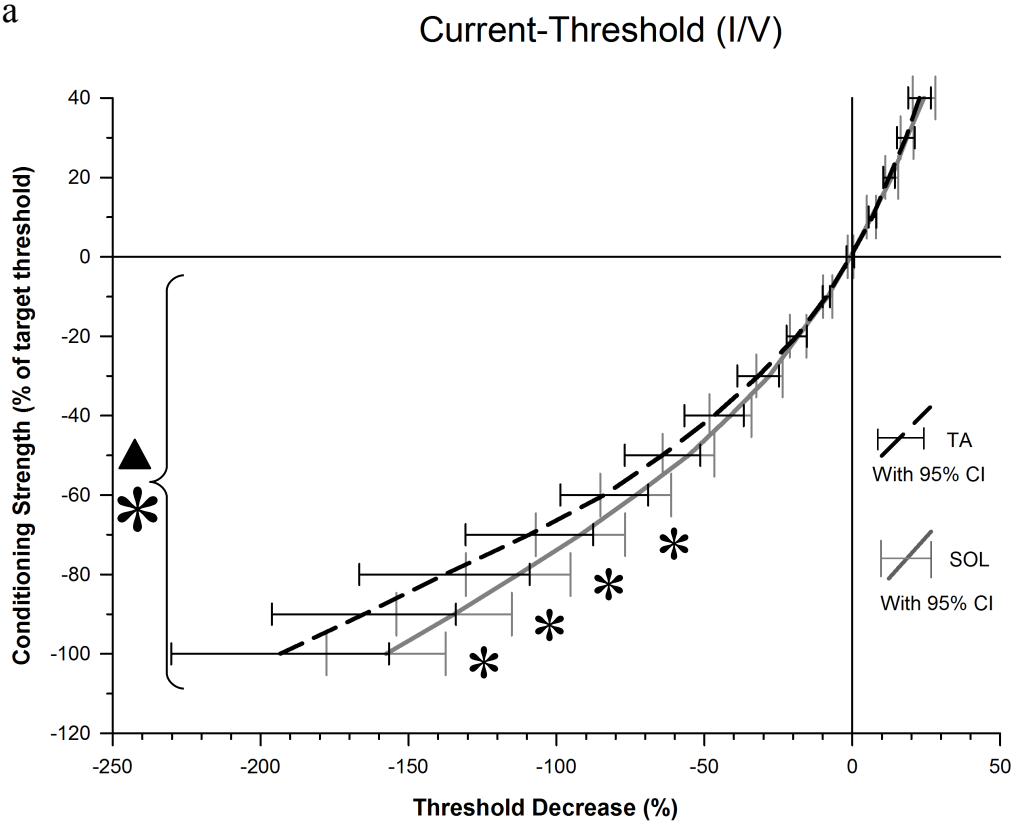
4.1.4 Current-Threshold

I/V is the only AET measure which uses conditioning current strength as an independent variable. The slope of this graph is strongly related to axon ionic conductances. At depolarizing conditioning strengths (the upper right quadrant of Fig 4.4a), the threshold decrease is similar for TA and SOL axons. There was no significant main effect of axon group ($F_{1, 8} = 0.965$, $p = 0.36$) nor a significant interaction effect between axon group and conditioning strength ($F_{2.31, 18.44} = 2.43$, $p = 0.11$) in depolarizing I/V. Note that the dependent variable, threshold reduction, is plotted on the x-axis in both Figs 4.4a and 4.4b, which is in accordance with plotting convention for I/V (see Maurer *et al.* 2007 and Krishnan *et al.* 2009, for example).

As the depolarized conditioning strength grows, a steepening slope in I/V

generally indicates that the there is increasing input conductance due to outward rectification on the axon (which is in turn resulting from an increase in ionic conductances such as fast and slow K^+ conductances). The slope is seen to be increasing in a similar fashion for both TA and SOL axons in the upper right quadrant of Fig 4.4a, therefore showing similar degrees of outward rectification in TA and SOL axons.. The slopes of the TA and SOL I/V curves were calculated (using secant lines between conditioning strengths separated by 20%) and a repeated measures analysis of these slopes did not reveal a significant main effect of axon group ($F_{1, 8} = 2.19$, $p = 0.18$) nor a significant interaction effect ($F_{1.74, 13.95} = 1.95$, $p = 0.18$) at depolarizing conditioning strengths. In other words, the input conductance of TA and SOL axons is similar at depolarized conditioning strengths in I/V. For simplicity in Fig 4.4b, we did not plot these depolarized slopes, but instead chose to plot the slopes of the TA and SOL curves at hyperpolarizing conditioning strengths only. On the lower left quadrant of Fig 4.4a, where axons are being subjected to incrementally increasing hyperpolarizing conditioning pulses, SOL axon responses are clearly different from TA axon responses, whereby SOL axons have a smaller increase in threshold than TA axons at the majority of the hyperpolarizing conditioning strengths. Although the rate of change of slope is decreasing as the strength of hyperpolarization grows in both TA and SOL axons (*i.e.* when comparing the curve at the right vs left sides, the slope is decreasing at a slower rate on the left), there was actually no *true* inward rectification found in both axon groups under investigation.

a


Current-Threshold (I/V)
Conditioning Strength (% of target threshold)
Threshold Decrease (%)
TA
With 95% CI
SOL
With 95% CI

b

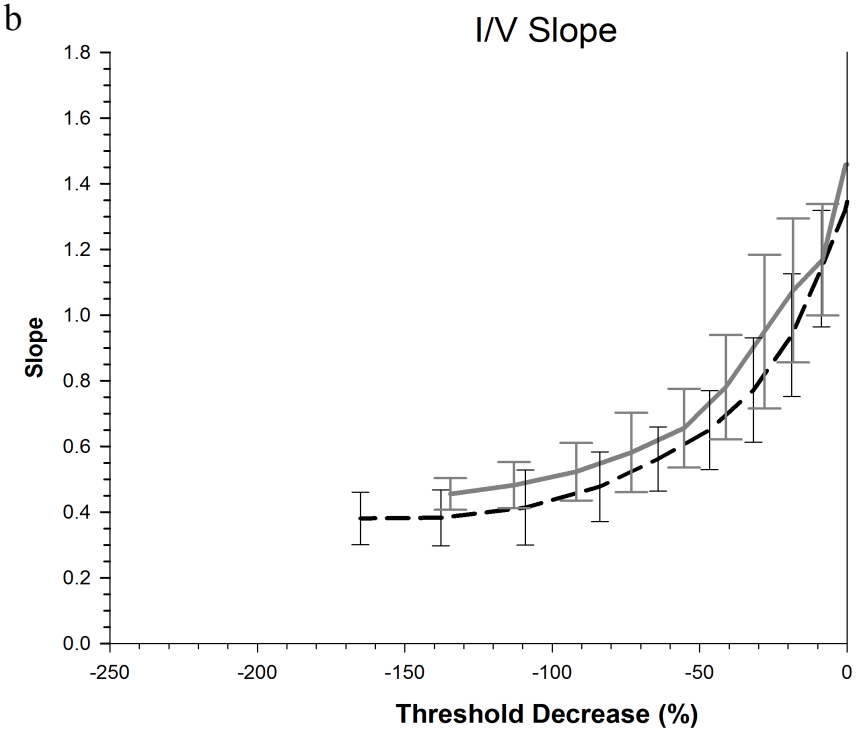

I/V Slope
Slope
Threshold Decrease (%)

Figure 4.4. Threshold decrease in TA axons was significantly smaller than in SOL axons at hyperpolarized levels in I/V. Correspondingly, the slopes from each curve in the bottom left quadrant of (a) were calculated, as shown in (b). The dependent variable (threshold decrease) is plotted on the x-axis in both plots. We have followed AET convention by displaying I/V and its slope in this manner. There was one between-subjects factor (rat) and two within-subjects factors (axon group and conditioning strength) in each of the repeated measures analyses. a) At hyperpolarizing conditioning strengths, a significant main effect of axon group ($F_{1, 8} = 8.14$, $p = 0.021$) and also a significant interaction effect between the two within-subjects factors ($F_{1.25, 9.97} = 9.95$, $p = 0.008$) were found and are indicated on the plot by the large asterisk (*) and a black-filled triangle (▲), respectively. The Bonferoni post-hoc tests found significant differences at individual conditioning strengths of -70%, -80%, -90%, and -100%, which are indicated by small asterisks (*) at the corresponding conditioning strengths (see text for more details). These findings show that the threshold increase for TA axons is larger than the threshold increase for SOL axons at hyperpolarized levels in I/V. b) The slopes of each I/V curve at hyperpolarizing conditioning strengths from (a) were calculated, and a repeated measures test did not find a significant main effect of axon group ($F_{1, 8} = 4.97$, $p = 0.056$) nor a significant interaction effect ($F_{2.13, 17.03} = 1.04$, $p = 0.38$). See text for details on the relationship between the interaction effect in I/V and the main effect in I/V Slope. The lack of a significant interaction effect in I/V Slope indicates that the change in slope, or conductance flux, is similar for TA and SOL axons across the varying strengths of hyperpolarizing conditioning pulses.

By true inward rectification we mean that which is normally observed in the I/V tests of humans and rodents; namely, there is normally an inflection point in the I/V curve as the hyperpolarizing conditioning strength increases (*i.e.* moving from right to left in the lower left quadrant of Fig 4.4a), and the slope begins to increase after this inflection point as a result of inward rectification. Although there is no inflection point apparent in this I/V plot, it can be seen in Fig 4.4b that the tangent to the slope is nevertheless decreasing as threshold increases, in both TA and SOL axons. It is possible that if we had used larger hyperpolarizing conditioning strengths that an eventual increase in slope and thus true inward rectification would have been observed in both TA and SOL axons. In either case, when moving from right to left in each of the graphs it is clear that the differences in slope in Fig 4.4b (which first visibly occur at a threshold increase of around

20% on the x-axis in Fig 4.4b) are associated with the differences in threshold of TA and SOL axons (which first visibly occur at a threshold increase of 30% on the x-axis in Fig 4.4a). These both hint at possible differences in the degree of inward rectification between TA and SOL axons at hyperpolarizing conditioning strengths. Check the figure legend for results from the statistical comparisons of TA and SOL axons at hyperpolarized conditioning strengths in I/V.

Repeated measures analysis of I/V at hyperpolarized conditioning strengths (lower left quadrant of Fig 4.4a) confirmed what the plot suggested – there was both a significant main effect and a significant interaction effect found (Fig 4.4a). This analysis included all 10 levels of hyperpolarizing conditioning strengths. Bonferoni post-hoc comparisons determined that TA threshold decrease was significantly smaller than SOL threshold decrease at conditioning strengths of -70% (T_{16} = -2.80, p = 0.01), -80 % (T_{16} = -4.03, p < 0.001), -90 % (T_{16} = -4.94, p < 0.001), and -100% (T_{16} = -5.80, p = < 0.001). The significant interaction effect in I/V is perhaps the simplest interaction effect to explain physiologically among the interaction effects in the 3 AET measures which explored this. Essentially, the interaction effect in I/V is measuring the same physiological phenomenon as the main effect in I/V Slope (these results are given in the Fig 4.4 legend). Both are comparing differences in the slopes of the TA and SOL I/V curves – although it is not an explicit investigation of slope, the interaction effect in I/V is simply comparing the differences between the change in threshold decrease (*i.e.* the slope) of TA and SOL axons across multiple conditioning strengths. Testing for an interaction effect in I/V was apparently

more sensitive to differences in slope than testing for a main effect in I/V slope. Note that main effect of axon group in I/V Slope had a p-value just above 0.05, however. The significant main effect as well as the significant interaction effect in I/V both provide similar evidence that TA axons tend towards having less inward rectification than SOL axons at hyperpolarizing conditioning strengths.

Whereas the interaction effects in TE and RC are assessing the interaction between delay and threshold change, in I/V the interaction effect does not have delay as a within-subjects variable. Therefore, the various statistical analyses related to I/V are measuring threshold properties *at a single point in time* (200 ms) after a conditioning pulse is applied. In summary, the results from I/V support the visual evidence from the plots that SOL axons have a greater propensity for inward rectification than TA axons at hyperpolarizing conditioning strengths.

4.1.5 Recovery Cycle

The recovery cycle (RC) offers impulse-dependent information about axons. Whereas TE and I/V tests examine changes in threshold occurring from subthreshold stimulation, RC assesses the oscillation of axon threshold which occurs after supramaximal stimulation of the TA or SOL group of axons. It should be noted that each of the measurements (displayed below in Fig 4.5) throughout the 200 ms recording window in RC indicate threshold changes for axons which produce a $CMAP_{40\%}$.

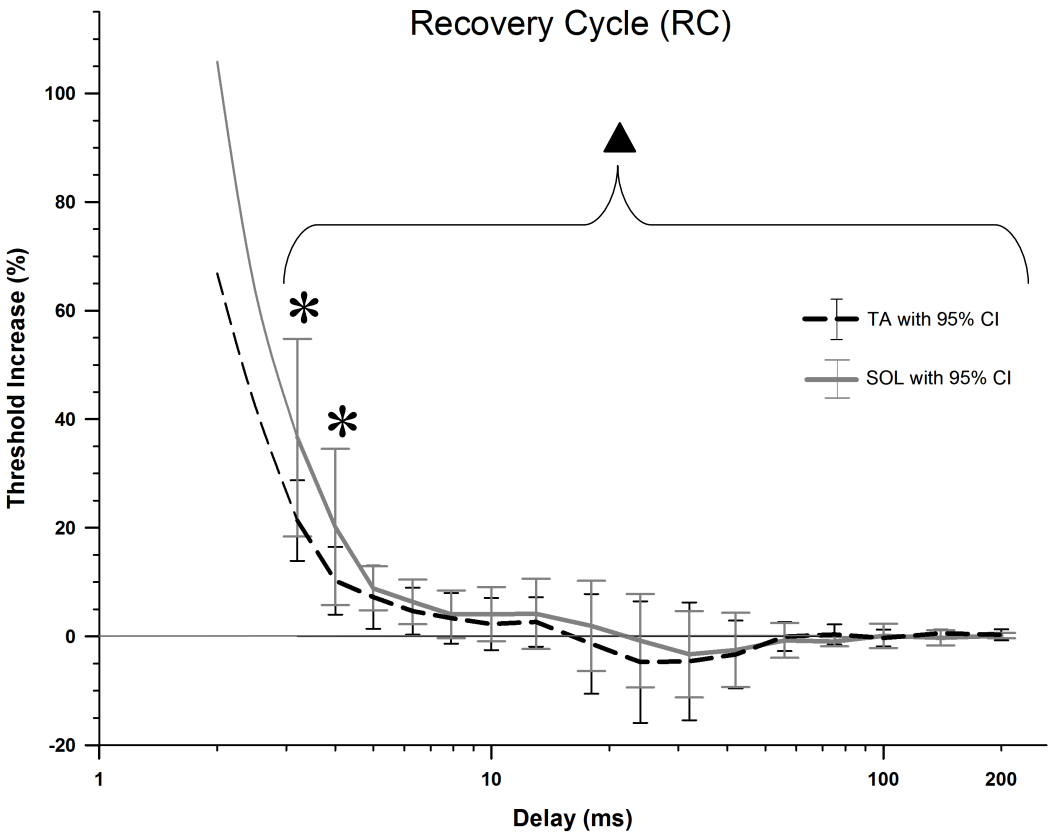


Fig 4.5. RCs of TA and SOL were compared by a repeated measures analysis. There was no significant main effect of axon group ($F_{1, 6} = 2.87$, $p = 0.14$) in RC. However, there was a significant interaction effect between the factors, axon group and delay ($F_{2.58, 15.49} = 3.94$, $p = 0.033$), indicating that after an impulse is fired, TA axon threshold fluctuates in a significantly different way than SOL axon threshold. Bonferoni post-hoc comparisons found TA axon threshold increase to be significantly smaller than SOL axon threshold increase at delays of 3.2 ms ($T_{12} = -5.98$, $p < 0.001$) and 4 ms ($T_{12} = -3.88$, $p < 0.001$). Note that the first two delays, 2 ms and 2.5 ms, are shown in this figure (thinner lines which are lacking a 95% CI), but they were not included in the statistical analysis because of missing SOL axon data from some rats at these two delays. Sample size was smaller in RC (n=7) than other AET measures. There was one between-subjects factor (rat) and two within-subjects factors (axon group and delay), which are identical factors in the repeated measures analyses of TE. The significant main effect and interaction effect are indicated as before with a large asterisk and black-filled triangle, respectively. The significant differences found by the Bonferoni post-hoc analysis are indicated by small asterisks.

When speaking of TA or SOL axons as "reaching the target CMAP", we are referring strictly to the firing of the *entire* subset of axons which produce the $CMAP_{40\%}$ (as opposed to higher threshold axons which produce the other 60% of

the electrically-recruited CMAP).

Before delving into the statistical results, it is useful to describe some general properties evident in Fig 4.5: (i) both TA and SOL axons approach the absolute refractory period as the delay between the supramaximal and test stimuli shortens to 2 ms, although SOL axon threshold is increasing at a faster rate than TA axon threshold. It is clear that as the delay shortens and approaches 2 ms, the threshold for SOL axons is approaching more of a vertical asymptote than TA axons and thus approaching the absolute refractory period. In this study, the SOL axons in 3 out of 8 rats failed to produce the target CMAP when the delay was 2 ms. It should be noted there are 8 instead of 9 rats here because in SOL axons in one rat RC was highly irregular and probably contaminated by noise, and therefore this data was discarded. It was found that TA axons in all 9 rats were able to produce the target CMAP at a delay of 2 ms. This highlights the conspicuous variability of absolute refractoriness. (ii) RRP is anomalous in our study because it is not interrupted by a period of superexcitability until very late in RC. Superexcitability refers to the point where axons become more excitable than normal and threshold drops below the x-axis. Other studies looking at RC in motor axons innervating the tail muscle in rats have observed a superexcitability interval which is comparable to that in humans (George and Bostock 2007, Mori *et al.* 2010). Peak superexcitability in these studies begins around 2 to 3 ms and ends no later than 30 ms. In our averaged results, we have found that superexcitability in both TA and SOL axons does not begin until 18 ms and 24 ms in TA and SOL axons, respectively. Superexcitability ends after crossing above

the x-axis at just after 56 ms in TA axons and after 75 ms in SOL axons (Fig 4.5). Peak superexcitability in the motor axons of the papers cited above occurs around 5 ms, whereas in the average of the 9 rats seen in Fig 4.5, peak superexcitability ocurrs at 24 ms and 32 ms in TA and SOL axons, respectively. Furthermore, the amplitude of peak superexcitability was -13% in George and Bostock (2007) and -15% in Mori *et al.* (2010). This is much larger than the magnitude of peak superexcitability of -4.72 ± 4.57% in TA axons and -3.29% ± 3.25% seen in the present results. It should be noted that superexcitability has been found to vary according to the muscle innervated: one study found axons innervating mouse plantar muscles to have a superexcitability near 0%, which was comparably smaller than the magnitude of superexcitability of axons innervating the mouse tail muscles in the same study, which was greater than 10% (Boerio *et al.* 2009). Overall, superexcitability in the present RCs of TA and SOL axons is peculiar in its timing, and perhaps in its magnitude as well.

(iii) Subexcitability, a period of reduced threshold, normally occurs after superexcitability when axon threshold in RC becomes greater than the unconditioned threshold and and moves above the x-axis . In Fig 4.5, however, it is hard to see any subexcitability. In fact, TA axon threshold is above the x-axis at 75 ms (where threshold increase is 0.36 ± 0.76%), but then goes below the x-axis again at 100 ms (where threshold increase is -0.29 ± 0.64%), only to be above the x-axis again for the last two delays of 140 ms and 200 ms (where the maximum threshold increase is 0.57% ± 0.30%). The alternation above and below the x-axis (*i.e.* above and below the unconditioned threshold), as well as the very small

values seen, indicates that subexcitability is missing in TA axons. In SOL axons, there is a similar alternation seen in the threshold going above and below the x-axis when the superexcitability ends after 75 ms. The maximum increase in threshold after superexcitability in SOL axons occurs at the very last delay (200 ms) and is only 0.14 ± 0.20%, indicating that subexcitability is also missing in SOL axons. Subexcitability has been measured in previous studies on rats and a maximum of 4.7 ± 1.8% (mean ± SD) was found at about 40-50 ms in rat tail motor axons in one study (George and Bostock 2007) while another study found a maximum of 2.0 ± 1.4% (mean ± SD) to occur at about 30-50 ms in the tail motor axons of immature and mature rats (Mori *et al.* 2010). Both of these studies used percutaneous stimulation and intramuscular needle recordings, the same techniques used in the present study, thus providing a large contrast to our present results on subexcitability.

The RC plot for TA and SOL in Fig 4.5 shows that TA axon threshold increase is smaller than SOL axon threshold increase for delays between 2 ms and 56 ms. A repeated measures analysis which included all delays between 3.2 ms and 200 ms did not find a significant main effect of axon group and therefore, overall, it is found that TA axons do not have a significantly different threshold change than SOL axons in RC. As stated earlier, the first two delays normally included in RC, 2 ms and 2.5 ms, were not included in the repeated measures analysis because of missing data from SOL axons. At a 2 ms delay, the SOL axons from 3 out of 8 rats were unable to produce the target CMAP (the threshold current required to produce the CMAP exceeded that allowed by our stimulator).

At a 2.5 ms delay, SOL axons from 2 out of 8 rats were unable produce the target CMAP. By 3.2 ms delay (the earliest delay used in the repeated measures analysis), SOL axons from 1 rat still could not produce the target CMAP. In contrast, TA axons from all 9 rats were able to produce the target CMAP at 2 ms and beyond. Though we did not statistically analyze absolute refractory period, the missing data from SOL axons attests to the likelihood that SOL axons have a larger absolute refractory period than TA axons. Also, because the repeated measures analysis was prevented from including 2 ms and 2.5 ms, there is an experimental bias introduced which prevents repeated measures analysis from detecting potential differences between TA and SOL axon thresholds at the first two delays in RC.

There was a significant interaction effect between the within-subjects groups, axon group and delay. Statistically speaking, the interaction effect means that the axon group (TA vs. SOL) determines the way in which threshold will oscillate in RC. In other words, it indicates that from 3.2 ms to 200 ms delay in RC, the rates of threshold change are significantly different for TA axons compared to SOL axons. It is important to note that, in contrast to the grouped delays used in TE, the repeated measures analysis in RC used measurements made at sixteen delays. All sixteen delays were used because there are no standards in AET literature from which to choose delays from in RC, other than where superexcitability peaks and sometimes where subexcitability peaks (see Park *et al.* 2009, Trevillion *et al.* 2010, Mori *et al.* 2010, Boerio *et al.* 2009, Kanai *et al.* 2006 and Vucic & Kiernan 2006, for example). Since in the present study there is

an abnormal superexcitability observed, and since subexcitability is lacking, we opted to not have post-hoc comparisons at specific delays. Interpretation of the interaction effect in RC is relatively complicated because there are 16 levels of the delay factor (compared to the TE analysis in which there are only a maximum of 3 levels of the delay group factor), therefore a question to be addressed in Chapter 5 is what, if any, are the physiological underpinnings of the interaction effect in RC.

4.1.6 Rheobase and Strength-Duration Time Constant

Figure 4.6 displays the results derived from 5 threshold measurements made at 5 different pulse durations, for both TA and SOL axons. In Fig 4.6a the dependent variable is expressed in terms of *current*, while Fig 4.6b has the dependent variable expressed in terms of *charge*. Therefore, Figs 4.6a and 4.6b are simply different depictions of the same data. It can be seen from Fig 4.6b that there is linear relationship between threshold charge and pulse duration. A linear regression on the data from Fig 4.6b can determine the slope of the line, which is numerically represented by rheobase in Fig 4.6c. This linear regression also determines the negative x-intercept of the line, which is numerically represented by SDTC (Fig 4.6d). See section 2.4.2.5 for more information. An independent samples t-test was carried out to determine if rheobase was significantly different between TA and SOL axons. Likewise, an independent samples t-test was carried out to determine if SDTC was significantly different between TA and SOL axons. Neither of these AET measures were found to be significantly different between TA and SOL axons.

Figure 4.6c displays the boxplot of our findings for rheobase. Rheobase is the only measure which is not unique to AET, although it is more commonly recorded at the MN soma rather than at the axon. Rheobase represents the estimated threshold current for an infinitely long stimulus. Since there is really only one independent variable, axon group, an independent samples t-test is ideal for comparing TA and SOL axon rheobase. An independent samples t-test did not find a significant difference between the two axon groups. Rheobase is the most sensitive of the AET measures to experimental variables such as the stimulating electrode placement, body temperature, and tissue impedance. Rheobase was found to be significantly larger in TA axons compared to SOL axons.

SDTC reflects the rate at which threshold current decreases as the stimulus duration decreases from 1.0 to 0.2 ms. As is the case with rheobase, there is only one independent variable in this measure (axon group) and so an independent samples t-test is ideal for comparing TA and SOL axon SDTC. It was determined that TA axons do not have a significantly smaller SDTC than SOL axons. Thus, as stimulus duration increases from 0.2 ms to 1.0 ms, TA axon threshold current decreases in a similar way to the SOL axon threshold current decrease. SDTC is thought to correlate with certain ion channel conductances, and so we will deal with the implications of this nonsignificant finding in the next chapter.

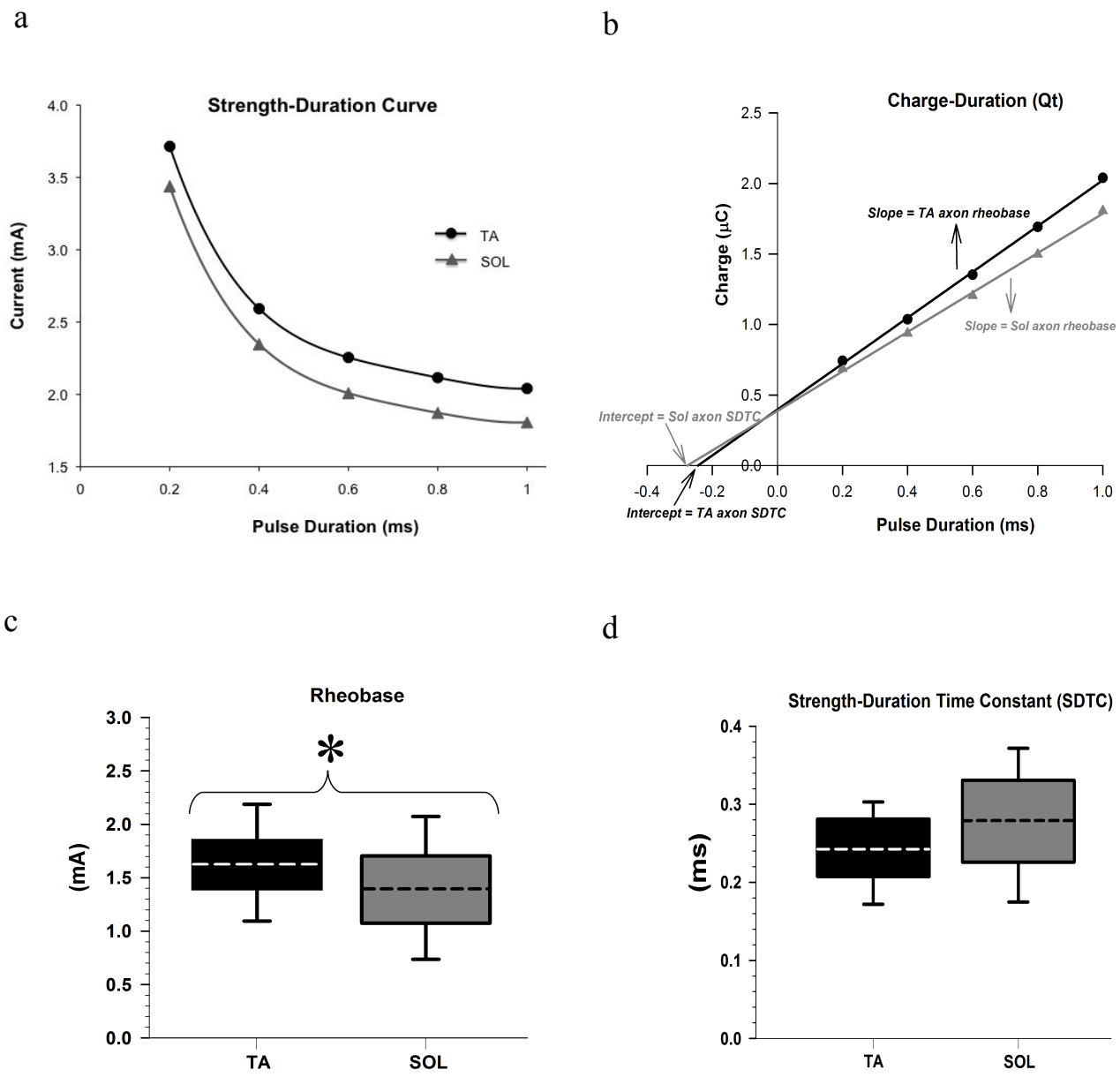


Figure 4.6. a) Threshold measurements made at 5 different pulse durations shows a relationship between threshold current and pulse duration that approximates an exponential curve. The shape of this "strength-duration" curve appears to be relatively similar for TA and SOL axons. b) The same data from (a) can be expressed in terms of charge rather than current, which reveals a linear relationship between threshold charge and pulse duration. A linear regression on the averaged Qt data from TA axons (n=9) gives a linear relationship of Q = 0.39 + 1.63*t, where Q is charge and t is pulse duration. A linear regression on the averaged Qt data from SOL axons (n=9) gives a linear relationship of Q = 0.39 + 1.40*t. Rheobase and SDTC are determined by these linear regression equations. c) A paired samples t-test found that the rheobase of TA (black-filled boxplot, 1.63 ± 0.11 mA) and SOL axons (grey-filled boxplot, 1.40 ± 0.14 mA) was significantly different (T_8 = 2.97; p = 0.020). d) SDTC was not significantly different between TA (black fill, 0.24 ± 0.01 ms) and SOL axons (grey fill, 0.28 ± .02 ms), as determined by an independent samples t-test (T_{16} = -2.08, p = 0.070).

4.2 Comparison of Anaesthetics

We originally had measurements from 14 rats in our dataset, with 5 of the 14 rats given sodium pentobarbital as an anaesthetic whereas the rest were given ketamine/xylazine. A repeated measures analysis is not an appropriate statistical comparison to use since there are two between-groups factors, "rat" and "anaesthetic", rather than just one between-groups factor. Our data failed to satisfy the assumption of equal variances, which is required in a two-way ANOVA. To our knowledge, when this assumption is violated, SPSS provides no alternative F statistic to the one provided by a normal two-way ANOVA. We therefore used independent samples t-tests to investigate differences between the two anaesthetics, and in the few cases where Levene's test showed the assumption of equal variances to be violated, we used a modified t-test which lowers the number of degrees of freedom and which therefore gives a larger p-value (this alternative p-value is automatically provided by SPSS in every independent samples t-test). This option provided by SPSS allowed us to compare the anaesthetics using independent samples t-tests even though our data violated the assumption of equal variances, and was thus useful in limiting Type I statistical errors.

In spite of the initial assumption that there would be no difference between these two anaesthetics, since they are known to primarily affect processes at CNS synapses rather than voltage-gated ion channels along a peripheral motor axon, it became apparent that there were differences in the responses to the two anaesthetics in some of the AET measures. After correcting for multiple

comparisons (through Bonferoni corrections in each of the AET measures except rheobase and SDTC, which do not need a Bonferoni correction), significant differences were found at specific delay groups in RC and in some TE tests, for both TA and SOL axons. Because of the significant differences between the two anaesthetics used in this study, we were required to eliminate the confounding variable, anaesthetic, in our comparisons between TA and SOL axons. We therefore had the option of excluding all measurements from either the ketamine/xylazine rats or sodium pentobarbital rats. Since more data was available from rats given ketamine/xylazine, the data from sodium pentobarbital rats was excluded. Thus, throughout section 4.1 above, the results only include data from ketamine/xylazine rats. We will now briefly share some of our comparisons between sodium pentobarbital and ketamine/xylazine AET measurements, as well as a few implications this has on our comparison of TA and SOL axons. RC results are presented first, as they present the most marked visual contrast between sodium pentobarbital and ketamine/xylazine. TE results are shown after.

4.2.1 Recovery Cycle

In addition to the statistically significant differences in the effects of sodium pentobarbital and ketamine/xylazine on RC (see figure 4.7 legend), a few observations from the RC of TA vs. SOL axons seem worthy of mention. First, it is readily seen that the average RC for sodium pentobarbital, for both TA and SOL axons, has a superexcitability period (ranging from approximately 4 ms to 10 ms in TA axons and from 5 to 7.9 ms in SOL axons) as well as a subexcitability

period (ranging from approximately 10 ms to 140 ms in TA axons and from 7.9 ms to 100 ms in SOL axons). The RC findings from rats that were given sodium pentobarbital, therefore, are much more in line with previous findings for RC in rats (see George and Bostock 2007, Mori *et al.* 2010). This is in contrast to the RC of rats given ketamine/xylazine in our study, which had an odd superexcitability that was quite late, as well as no clear subexcitability. Also, for the RC in rats given sodium pentobarbital, the average threshold increase at the earliest delay of 3.2 ms was 7.56 ± 18.49% in TA axons (mean ± 95% CI – Fig 4.7a). This average threshold increase is lower than that for SOL axons at a 3.2 ms delay, which was 31.13 ± 16.29% (Fig 4.7b). Maximum superexcitability occurred at a delay of 6.3 ms in both TA and SOL axons of rats given sodium pentobarbital, where the average threshold increase was -9.36 ± 7.16% and -2.74 ± 3.91 %, respectively. Maximum subexcitability occurred at 24 ms in both TA and SOL axons, and was 19.09 ± 28.76% and 26.63 ± 25.12%, respectively, in rats given sodium pentobarbital. Although no statistical tests were carried out that compared TA and SOL axons from rats given sodium pentobarbital (due to the very small sample sizes and the corresponding low statistical power), these preliminary findings from sodium pentobarbital rats support the evidence in section 4.1.5 that the excitability of TA axons oscillates differently than SOL axons in RC. These findings also point to the possibility that TA axons have less of a threshold increase (or greater excitability) than SOL axons at certain delays in RC.

Since the sample size for rats given sodium pentobarbital was very small

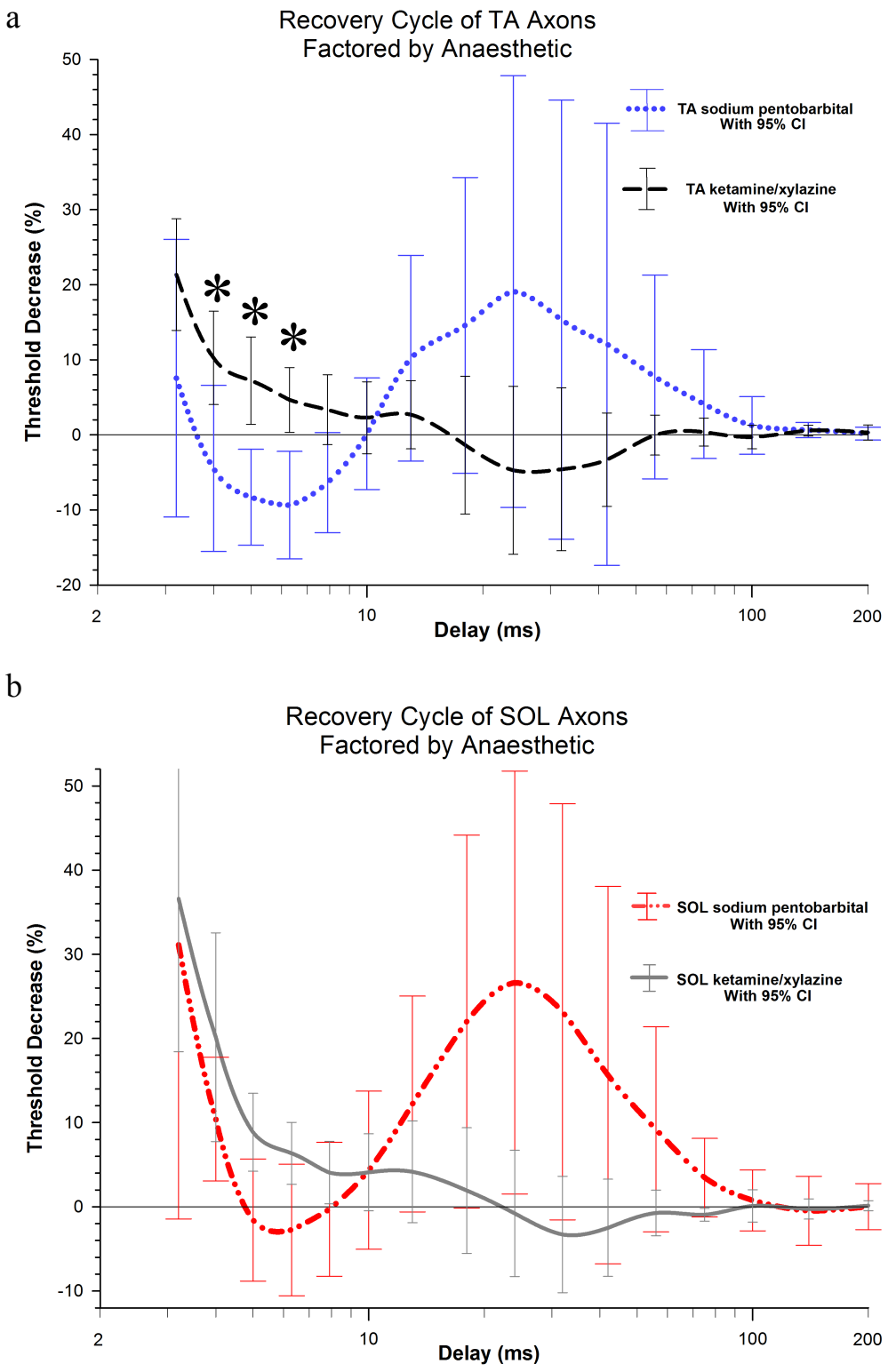


Figure 4.7. Comparison of RC from rats given sodium pentobarbital with the RC from rats given ketamine/xylazine. a) The TA axon RC from rats given sodium pentobarbital (n=5) was compared to the TA axon RC from rats given ketamine/xylazine (n=9), at all sixteen delays ranging from 3.2 ms to 200 ms. Despite the small sample size and the small p-value required for familywise significance ($p < 0.003$) under the Bonferoni

correction for sixteen comparisons, independent-samples t-tests found significant differences between sodium pentobarbital and ketamine/xylazine at delays of 4.0 ms (T_{12} = -3.86, p = 0.002), 5.0 ms (T_{12} = -4.99, p < 0.0005), and 6.3 ms (T_{12} = -4.99, p ≤ 0.0005). These are indicated by asterisks (*) at the respective delays. b) The RC for SOL axons from rats given sodium pentobarbital was compared to the RC for SOL axons from rats given ketamine/xylazine through independent samples t-tests, in the same way done for TA axons. For SOL axons, no familywise significant differences between the two anaesthetics were found.

(n=5), the statistical power of any comparisons involving these rats was correspondingly small, and it is likely that more differences would have been detected between the RCs of the two anaesthetics had our sample sizes been larger. Overall, the qualitative aspects of the RCs for both TA and SOL axons from rats given sodium pentobarbital are much more similar to motor axon RCs published in the literature than the RCs from rats given ketamine/xylazine. The previous findings in section 4.1.5 regarding the RCs of TA and SOL axons from rats given ketamine/xylazine, therefore, are shown to be somewhat less reliable than RC comparisons in sodium pentobarbital rats.

4.2.2 Threshold Electrotonus

Specific delay groups from TA and SOL axon TE from rats given ketamine/xylazine have already been analyzed and displayed in Fig 4.3; now, part of this data is being compared to the TE of rats given sodium pentobarbital. Part of the data is shown in Fig 4.8 below. Specifically, TA axons from sodium pentobarbital rats were compared to TA axons from ketamine/xylazine rats, in all four TE tests, using independent samples t-tests. Differences between the two anaesthetics in SOL axons were similarly analyzed. After adjusting the p-value for multiple comparisons

(six in each TE test) using the Bonferoni correction, significant differences between the two anaesthetics were found at some delay groups.

Overall, these TE results show that sodium pentobarbital produces a more fanned-out waveform (i.e. the absolute magnitude of threshold change values are larger) compared to ketamine/xylazine in all TE tests, for both TA and SOL axons. In section 4.1.3.1, which compared TA and SOL axons using ketamine/xylazine rats, the early delay group (20-30 ms) was excluded in hyperpolarized TE analyses because it is not usually analyzed in the literature. This was analyzed in the present comparison between anaesthetics, however, since other AET measures (*i.e.* RC and all TE tests) indicated that sodium pentobarbital and ketamine/xylazine caused differential responses at early delays. The threshold decrease in TA TEd +20% at 20-30 ms was 25.22 ± 0.94% for sodium pentobarbital and 17.84 ± 0.95% for ketamine/xylazine. In TA axons, the threshold decrease in TEh -20% at 20-30 ms was -26.66 ± 1.44% for sodium pentobarbital and -20.02 ± 0.75% for ketamine/xylazine. For SOL axons in TEh -20% at 20-30 ms threshold decrease was -24.43 ± 0.91% for sodium pentobarbital and -19.10 ± 0.75% for ketamine/xylazine. In addition to these familywise significant differences shown in Fig 4.9, there were also familywise significant differences between the anaesthetics in other TEd and TEh measures. For TA axons, there was a significant difference in TEd +40% at 20-30 ms (T_{12} = 5.29, p = 0.001), as well as a significant difference in TEh -40% at 20-30 ms (T_{12} = 5.29, p = 0.002). For SOL axons, there were no

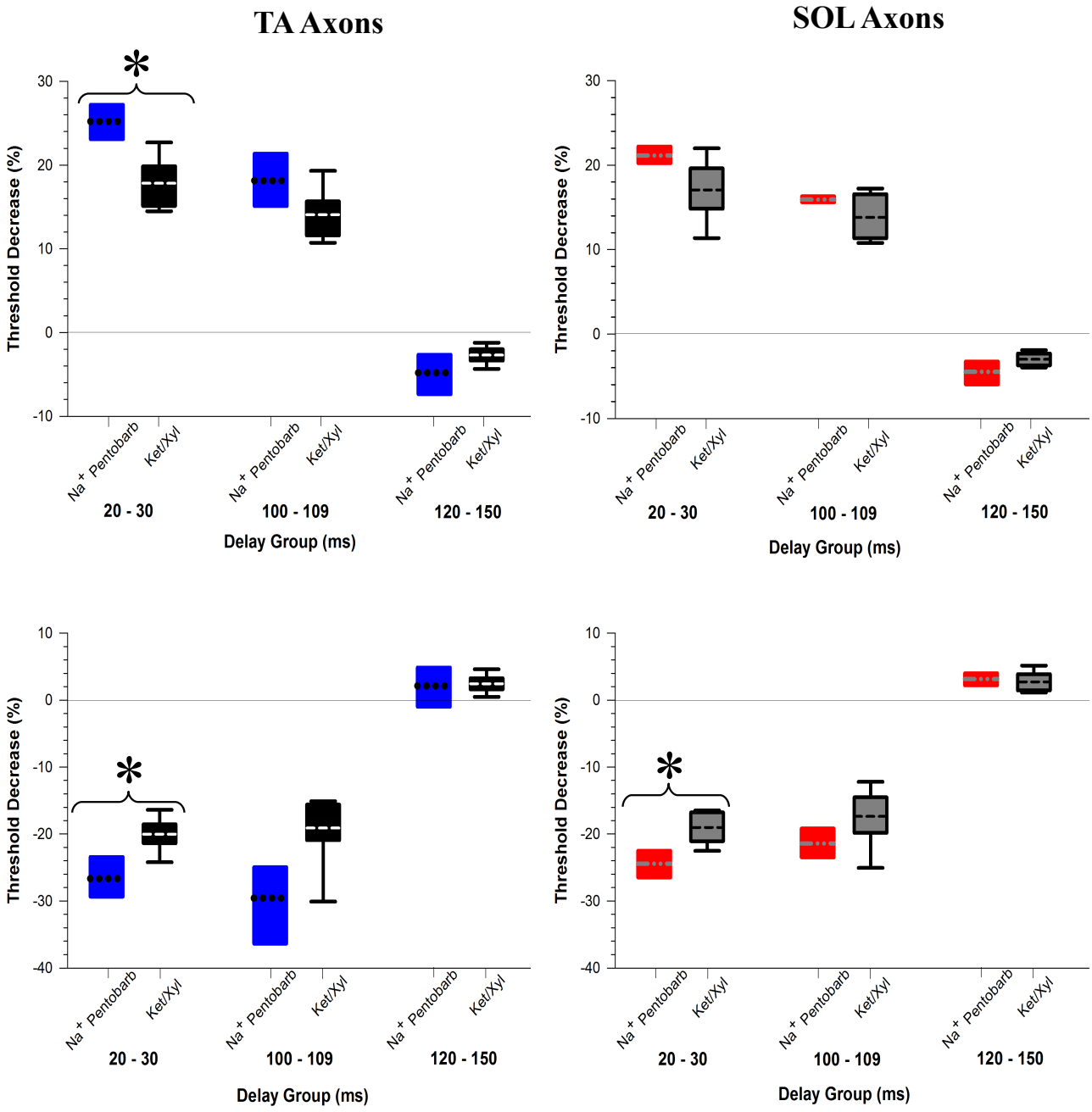


Figure 4.8. Comparisons between sodium pentobarbital rats (n=5; blue-filled and red-filled boxplots for TA and SOL axons, respectively) and ketamine/xylazine rats (n=9; black-filled and grey-filled boxplots for TA and SOL axons, respectively) via independent samples t-tests showed significant differences between the anaesthetics in both TA axons (left panels) and SOL axons (right panels), in TEd +20% (top panels) and TEh -20% (bottom panels). For TA axons, there were significant differences between sodium pentobarbital and ketamine/xylazine in TEd +20% at 20-30 ms ($T_{12} = 5.05$, $p < 0.0005$),

and in TEh -20% at 20-30 ms (T_{12} = -4.56 p = 0.001). For SOL axons, there were significant differences between anaesthetics in TEh -20% at 20-30 ms (T_{12} = -4.37, p = 0.001). These are significant at the Bonferoni familywise level and are indicated by asterisks (*) over the corresponding delay groups. The boxplots from ketamine/xylazine rats have been displayed previously in figures 4.3b and 4.3d, and are shown again here to facilitate visual evidence of the differential effects of the anaesthetics on rat TA and SOL axon TE. No whiskers were produced on the sodium pentobarbital boxplots due to their small sample size.

familywise significant differences between the anaesthetics in TEd +40% and TEh -40%.

Although they did not reach familywise statistical significance, there were individual significant differences between the anaesthetics (where $p < 0.05$) found at the delay groups, 100-109 ms and 120-150 ms, in all of the TE tests. For TEd +40%, individual significant differences occurred at 120-150 ms in both TA and SOL axons. For TEh -40%, individual significant differences occurred at 100-109 ms in TA axons. For TEd +20%, individual significant differences occurred at 100-109 ms in TA axons and at 120-150 ms in SOL axons. For TEh -20%, individual significant differences occurred at 100-109 ms in TA axons. We emphasize that these differences did not reach familywise significance under the Bonferoni correction. Nevertheless, these findings assist in illustrating how sodium pentobarbital and ketamine/xylazine probably produced different motor axon responses in multiple AET measurements. It was in light of these discoveries that we excluded data from the sodium pentobarbital rats in our comparisons of TA and SOL axon AET (section 4.1).

Chapter 5

Discussion

5.1 Differences in I_H Activity Between Fast and Slow Motor Axons

The significant main or interaction effect in hyperpolarizing TE and I/V both indicate that the activity of hyperpolarization-activated inwardly rectifying cation conductance (I_H) is greater in slow compared to fast motor axons (see sections 2.4.4, 2.4.6.2, 4.1.3, and 4.1.4). I_H is found in all 3 major neuron compartments: the soma, dendritic tree, and the axon proper (Baker *et al.* 1987, Takigawa *et al.* 1998, Robinson and Siegelbaum 2003, Biel *et al.* 2009). The role of I_H in various cells is very diverse, and unfortunately it has been studied in the neuron soma far more than it has been studied in the dendritic tree or axon proper. The most well-known function of I_H is in cardiac cells where this conductance, also known as funny or queer current, gives rise to spontaneous pacemaker activity (Robinson and Siegelbaum 2003). The role of I_H in neuropathic pain has recently gained a lot of attention as well (Carlton 2009). In regard to axons, there are two main

physiological roles of I_H: control/limitation of resting membrane potential and control of membrane resistance (Pape *et al.* 1996, Robinson and Siegelbaum 2003, Howells *et al.* 2012). A shared feature of I_H across all cells which contain this conductance is the self-limiting nature of I_H: activation of I_H causes depolarization which in turn leads to inactivation of I_H. The channel responsible for I_H , hyperpolarization-activated cyclic nucleotide-gated (HCN) channel, allows the passage of Na^+ and K^+ although it excludes Li^+.

There are four known isoforms of the HCN channel in humans, HCN1 to HCN4. HCN channels are typically activated at voltages of -50 to -60 mV or below, while the voltage of half-maximal activation ($V_{1/2}$) in neurons typically ranges from -60 mV to -100 mV. In adult heart ventricles, however, $V_{1/2}$ can be as low as -120 mV. Also see Howells *et al.* (2012) where axon computer simulations suggested HCN $V_{1/2}$ is -107 mV in human motor axons and -94 mV in human sensory axons. All HCN isoforms except HCN3 are regulated by direct binding to cAMP. Increases in intracellular cAMP can contribute to a positive shift of up to 20 mV in the voltage dependence of activation for I_H, with HCN2 and HCN4 generally having greater shifts in their activation curves (greater sensitivity to cAMP) than HCN1 (Robinson and Siegelbaum 2003, Biel *et al.* 2009). In addition to cAMP, I_H has been found to be influenced by acidic lipids (*i.e.* PIP2), protons, extracellular K^+ concentration, and cytosolic proteins, which interact with the HCN subunits (Biel *et al.* 2009). HCN1 has the fastest time constant of activation which can range anywhere from 30 ms to 200 ms at -140 mV to -95 mV (Biel *et al.* 2009). HCN2 is the next fastest isoform with the time constant of

activation ranging from 150 ms to 1 s (Biel *et al.* 2009).

Physiological information on HCN function in MN somas and motor axons is severely lacking. HCN1-3 isoforms have been found in mouse DRG MNs (Biel *et al.* 2009), although the distribution of these channels along peripheral motor axons remains unclear. Two recent studies comparing AET properties of motor and sensory axons found evidence for differential expression of HCN isoforms, with sensory axons possibly having greater expression of faster isoforms (*i.e.* HCN1) than motor axons (Howells *et al.* 2012, Nodera and Rutkove 2012). Interestingly, the variation in voltage-dependent properties of I_H seen across different cells in the heart is greater than the variation seen across the different isoforms of HCN, suggesting that the diversity of I_H is only partly attributable to HCN isoform diversity. Robinson and Siegelbaum (2003) have cautioned against rigid stereotyping of I_H behavior in terms of the HCN isoforms, and these authors have favoured the alternative viewpoint that I_H function in a particular cell or preparation is best understood by considering the interplay of other ionic currents and cellular constituents (such as cAMP and protein kinases) in addition to considering the stereotyped behavior of HCN isoforms. The viewpoint espoused by Robinson and Siegelbaum is thus opposed to understanding I_H behavior solely in terms of the behavior of the HCN isoforms. In short, the behavior of I_H is not only voltage-dependent but is also influenced by a variety of intracellular modulators such as cAMP, although the specific effects of I_H modulators i*n motor axons* have not been directly investigated.

In mammalian axons, the investigation of differential I_H expression has

only just recently begun. In the mid-1990s, Bostock and colleagues first detected the possibility of differential I_H expression between human motor and sensory axons using AET. This group found that motor and sensory axons differed only in their response to hyperpolarizing TE and not to depolarizing TE. Consequently, these motor-sensory axon differences in accommodation were attributed mainly to a differential expression of I_H (Bostock *et al.* 1994), although it is recognized that differences in other ion channels also exist between human motor and sensory axons (Vogel and Schwarz 1995). The finding of differential I_H expression has been supported and elaborated upon by additional studies using more advanced AET methodology as well as ischaemia to investigate differences between motor and sensory axons (Lin *et al.* 2002, Nodera and Rutkove 2012, Howells *et al.* 2012).

Through computer modeling, another study investigating differences between axons with different thresholds found that I_H appears to be more active at resting membrane potentials in the lower threshold axons than in the higher threshold axons (Trevillion *et al.* 2010). In fact, all of the above studies which have used AET to specifically investigate I_H in peripheral axons indicate that this conductance is active at the resting membrane potential. This finding is supported by the fact that I_H is active at resting membrane potential in a wide variety of other neuronal preparations (Biel *et al.* 2009). Modeling evidence indicates that I_H contributes noticeably to the axon resting membrane potential, and computer simulations from Howells *et al.* (2012) suggested that I_H partly contributes to a 4.0 mV depolarization of the resting membrane potential of sensory vs. motor axons.

Interestingly, there is also evidence that $V_{1/2}$ differs significantly between individual subjects. In their computer modeling, Howells *et al.* (2012) found $V_{1/2}$ to vary by 40 mV in motor axons and by 15 mV in sensory axons (with the average $V_{1/2}$ being -107 mV in motor axons and -94 mV in sensory axons). This inter-subject variability is supported by the previous AET study by Trevillion *et al.* (2010) which found that single motor axons were able to be classified into two groups on the basis of widely differing TEh (importantly, all other AET parameters in this study were not useful in distinguishing different types of axons).

Thus, it appears that I_H impacts the functioning of peripheral axons over a wide range of physiological conditions and not just during hyperpolarization. It also seems that the function of this conductance can vary widely between individuals, although there has been no investigation which has deeply examined the relationship of I_H to other peripheral motor axon properties. In the discussion section of Howells *et al.* (2012), it was asserted that inter-subject I_H variability could not be explained by cAMP levels alone, so other allosteric activators (*i.e.* PIP2) may also be involved in the regulation of the voltage properties of HCN channels and the activity of I_H in human motor axons. However, these authors also suggested the alternative possibility that a slower isoform of HCN may be responsible for motor-sensory differences. The suggestion, that different HCN isoforms are responsible for accommodation differences between motor and sensory axons, has been reiterated by Nodera and Rutkove (2012). Using AET on sensory axons of different thresholds, these researchers found a significant

correlation of threshold change to different delays in TEh (this correlation was not significant in motor axons of different thresholds). This correlation was ultimately used as evidence that sensory axons of different thresholds express multiple HCN isoforms with different kinetics, so that lower-threshold sensory axons have a greater amount of faster I_H through more HCN1 expression than higher-threshold axons. A lack of significant correlation in motor axons was used to infer that motor axons have only the faster HCN1 isoform (Nodera and Rutkove 2012).

Our present study supports the conclusion that there are physiological significant levels of the faster isoform, HCN1, in both TA and SOL axons (based on significant differences found between axons in rats given sodium pentobarbital vs. ketamine/xylazine – see below in section 5.7), although our analysis does not preclude the possibility that slower HCN isoforms such as HCN2 or HCN3 also exist on TA and SOL axons. From our comparison of TA and SOL axons in the TE and I/V measures we can infer that, in comparison to TA axons, SOL axons have greater activity of I_H . This is specifically because of the finding that SOL axons have statistically significant smaller excitability decreases in TEh -40% and TEh -20%, as well as statistically significant smaller excitability decreases in hyperpolarizing I/V, when compared to TA axons. On their own, the TE and I/V comparisons of TA and SOL axons do not present evidence for or against particular HCN isoforms being dominant in one type of axon, although as we will discuss below differences in the responses to our anaesthetics provide evidence that both axon groups contain the fastest isoform, HCN1.

The finding that there is a greater activity of I_H in slow compared to fast motor axons must be interpreted in light of the methods employed in this study. First, we must clarify that by stating "greater activity of I_H" we are not referring specifically to the number of HCN channels, the conductance of these channels, their distribution, or the electrochemical driving force behind the channels. Rather, we are referring to the interaction of all these factors which ultimately manifests in the ability of I_H to oppose hyperpolarizing currents and therefore oppose threshold increases. We are essentially stating that, when compared to fast motor axons, I_H in slow motor axons is able to more strongly oppose hyperpolarization (and the corresponding threshold increase) through an overall greater amount of inward cation current via HCN channels. The methodology employed in AET does not allow for distinctions to be made in regard to the underlying causes of this difference in I_H activity (in other words, AET cannot identify whether these differences in I_H activity are due to a difference in the number of HCN channels, a difference in HCN single-channel conductance, a difference in HCN distribution, or a difference in the electrochemical driving force, or some combination of these[1]). At present, nothing more can be proposed in regards to the exact physiological underpinnings which result in the greater activity of I_H in SOL axons (the terms " I_H activity" or " I_H expression" are used widely and interchangeably in AET literature - see Lin *et al.* 2002, Kiernan *et al.* 2004, Trevillion *et al.* 2010, Nodera and Rutkove 2012, Howells *et al.* 2012 for examples).

Axons innervating TA are mostly from fast motor units which fire at

higher frequencies than the axons of SOL which are mostly from slow motor units (Gillepsie *et al.* 1987, Totosy de Zepetnek *et al.* 1992, Gorassini *et al.* 2000, Kernell 2006). Activity-dependent hyperpolarization of axons is known to correlate positively with firing frequency as well as the with the number of impulses in a train (impulse load) (Erlanger and Gasser 1937, Raymond 1979, Morita *et al.* 1993, Kiernan *et al.* 2004), although the magnitude of hyperpolarization reaches a maximum at frequencies around 20-50 Hz in lizards (Morita *et al.* 1993) and possibly at 20-30 Hz in humans (Kiernan *et al.* 2004). Although the relative importance of firing frequency vs. impulse load in creating activity-dependent hyperpolarization in rat TA and SOL axons is unclear, from Raymond (1979) it is apparent that in the frog average firing frequencies as low as 1.25 Hz can cause axon threshold depression and therefore probably activity-dependent hyperpolarization (see Fig 9 in Raymond (1979) where 5 impulses separated by 10 milliseconds fire in a burst every 4 seconds). Bursts in rat SOL motor units fire at an average frequency of 20 Hz for roughly 30% of the day (Hennig and Lomo 1984), therefore it seems likely SOL axons would experience activity-dependent hyperpolarization via activation of the Na^+/K^+ pump, at various times throughout the day. Moreover, during rat locomotion, TA axons fire an average of 4.3 impulses per step cycle while SOL axons fire an average of 26 impulses per step cycle (although TA axons fire in shorter bursts at higher frequencies – see Gorassini *et al.* 2000), strengthening the possibility that during locomotion SOL axons have increased activation of the Na^+/K^+ pump, in comparison to TA axons.

We have found that TA axons possess lower levels of the accommodative current, I_H, than SOL axons. But why might this be? Since we know that rat SOL axons fire more impulses during each step than TA axons (Gorassini *et al.* 2000), and also since it is very likely that SOL axons fire more impulses each day compared to TA axons, the simplest explanation for differences in I_H between TA and SOL axons is the role of I_H in limiting activity-dependent hyperpolarization in SOL axons. Is it possible that this greater activity of I_H in SOL axons also promotes energy conservation in the cell? Energy conservation is unlikely to be a contributing factor for one main reason: since greater I_H causes a greater inward flow of cations, the majority of which are Na^+ ions at resting membrane potential (Robinson and Siegelbaum 2003, Biel *et al.* 2009), it is likely that the energy expenditure of the Na^+/K^+ pump is increased via increased I_H activity.

Another possible reason for greater activity of I_H in SOL axons may simply be greater levels of cAMP or other modulators in these axons. Alternatively, there may be additional factors other than membrane potential and axon energy expenditure which have led to differential I_H activity in motor axons. Increased I_H activity decreases axon membrane resistance and is believed, in certain preparations, to decrease the slowing of conduction velocity (CV) which is associated with RC subexcitability (Bucher and Goaillard 2011). At present there are no studies which have systematically tested the connections between I_H and CV in peripheral motor axons. It is quite possible that a complex web of causes and effects are associated with the differences in I_H between TA and SOL axons. There is thus far only one discernible effect of differential I_H expression in the

present study: in comparison to TA axons we see that SOL axons are more able to resist hyperpolarization through greater activity of I_H. The variability in I_H across different axons deserves further attention, and computer modeling may be needed to complement experimental investigations.

5.2 Recovery Cycle Findings

The findings from RC in section 4.1.5 show that the excitability oscillation, which occurs for approximately 200 ms after a single action potential is fired, is significantly different between TA and SOL axons. This makes some sense given that the firing patterns of TA axons are different than the firing patterns of SOL axons (Gorassini *et al.* 2000), and it is possible that the ionic conductances in each axon group have adapted uniquely to their own activity patterns. In table 2.1 and the associated text (section 2.4.4) we looked at previous reports of differences in AET parameters which were dependent upon the innervated muscle or the electrical recruitment threshold of the motor axons (Kiernan *et al.* 1996, Kuwabara *et al.* 2000, Kuwabara *et al.* 2001, Krishnan *et al.* 2004, Bae *et al.* 2009, Jankelowitz *et al.* 2009, Mori *et al*. 2010, Trevillion *et al.* 2010, Murray *et al.* 2011, Nodera and Rutkove 2012), although the degree to which ionic mechanisms are responsible for these differences is uncertain. In section 2.4.4.2 it was mentioned that it is likely that ionic conductances are at least partly responsible for differences in RC owing to the fact that much of the AET literature has demonstrated that ionic mechanisms affect each and every AET measure. We also explained in section 2.4.4.2 that the three prominent features of

RC – RRP, superexcitability, and subexcitability – are each affected by a unique combination of ionic conductances plus passive properties. However, we have only demonstrated a significant interaction effect and not a significant main effect in RC in this study, and so we have found no overall difference between TA and SOL axon excitability across the entire 200 ms window in RC.

It is perhaps revealing that, out of all the 12 pairs in table 2.1 which specifically investigated threshold-dependent differences in axons (comparisons *a, b, c, p, q, r, s, t, u, w, x*, and *y*), only two significant differences were found in RC, both of which were in the parameter, "subexcitability". Subexcitability has been shown to be influenced greatly by ionic conductances, mostly the slow K^+ conductance (section 2.4.4.2). Therefore, if axons of different electrical thresholds represent either (a) different sub-groups of motor axons with different active and passive membrane properties (this notion is supported by the idea that many AET parameters other than superexcitability are significantly different between motor axons of different thresholds), and/or (b) axons with different diameters (see Trevillion *et al.* 2010 for an AET study providing experimental evidence and a discussion of this idea), then it is possible that axon passive properties – mainly diameter - do *not* have a significant impact on RC. More importantly, superexcitability is believed to be the parameter within RC which is most directly influenced by passive membrane properties (although it is influenced by ionic conductances as well; see Barrett and Barrett 1982, Bostock *et al.* 1998, Krishnan *et al.* 2009), but it does not differ significantly in any of the 12 pairs of axon groups having different thresholds in table 2.1. If

superexcitability is highly dependent upon axon diameter, however, then a lack of significant findings for this parameter in table 2.1 is to be expected. This is because the axon pairs in each comparison from table 2.1 likely have greatly overlapping distributions in their diameters (cf. the modeling results from McIntyre *et al.* 2002, where it was proposed that an increasing axon diameter will produce a smaller superexcitability[2]). At any rate, a lack of significant findings for superexcitability does not contradict the idea that the other RC parameters are relatively insensitive to passive membrane properties.

Therefore, we consider it likely that ionic conductances have contributed to the significant interaction effect found in RC, as opposed to passive membrane properties, although we cannot pinpoint specific conductance(s) since there are multiple ion channels which influence RC. Though in the AET literature there is limited information regarding the effect of I_H on RC (reviews on AET such as Bostock *et al.* 1998 and Krishnan *et al.* 2009 did not include I_H as a conductance which influences RC; however, see Moalem-Taylor *et al.* 2007 for evidence that cesium-sensitive I_H influences superexcitability in C-fibers), it is possible that differential expression of I_H in TA and SOL axons has contributed to RC differences between these two groups.

When TA and SOL axons are completely faithful in their conduction of signals received by the MN soma, which is the prevailing assumption under healthy conditions, our RC findings bear no functional consequence for motor control. However, during axon dysfunction in certain peripheral nerve diseases these RC findings may be pertinent. Under certain disease conditions where there

is a risk of conduction block, TA axons may conduct signals more reliably than SOL axons. As can be seen in RC in Fig 4.5, mean TA axon excitability is larger than mean SOL excitability at certain delays. Although overall this difference in excitability was not statistically significant, the Bonferoni post-hoc tests showed that at early delays of 3.2 ms and 4.0 ms TA and SOL axon excitability are significantly different. In addition, the difference between TA and SOL axon excitability can be seen visually at early delays in RC since the 95% confidence interval for TA axons does not overlap with mean SOL axon excitability at these delays. Therefore, in some cases of axon dysfunction, it is possible that TA axons have a greater propensity for faithful conduction, especially at high firing frequencies.

The Bonferoni post-hoc tests performed at specific early delays in RC entail differences in axon ionic conductances which could foreshadow additional significant differences in RC between TA and SOL axons if *multiple action potentials* are fired. The post-spike excitability oscillation of an axon is highly dependent on the history of impulses, with certain impulse patterns generating a larger superexcitability and/or a larger subexcitability as well a temporal shift in the RC curve (Gasser and Erlanger 1937, Raymond 1979, Bucher and Goaillard 2011). Our results consequently raise the possibility that a significant main effect could be found between TA and SOL axon excitability if there were multiple action potentials preceding the conditioning pulse in RC rather than just one action potential. And since activation of TA and SOL axons usually involves bursts of several spikes rather than just two spikes (i.e. Hennig and Lomo 1985,

Gorassini *et al.* 2000), the scenario of having more than one conditioning pulse in RC is more realistic. Consequently, it seems quite feasible that in certain pathologies of peripheral nerve, the faithfulness of TA and SOL axons to reliably conduct action potentials could be significantly different. In summary, our RC findings provide some evidence that TA axons are likely to be more faithful than SOL axons in reliably transmitting signals to their target muscle fibers in these diseased states[3].

Also, as mentioned in section 2.2.4, short-term changes to CV normally occur in conjunction with the post-spike oscillations in axon excitability. For example, in mammalian myelinated axons an increased CV often occurs simultaneously with superexcitability in RC while a decreased CV often occurs simultaneously with subexcitability (Bullock 1951, Swadlow *et al.* 1980, Baker *et al.* 1987, Bucher and Goaillard 2011). The finding that TA axon post-spike excitability oscillation is significantly different than SOL axon post-spike excitability oscillation perhaps anticipates differences in the post-spike changes in CV between these two axon groups. Since from section 4.1.5 it is shown at certain early delays in RC TA axon excitability is larger than SOL axon excitability, it is possible that TA axons are better suited for high firing frequencies as well as high CVs after an action potential is fired. This "favoring" of TA axons towards higher CVs would be independent of axon morphology and could have implications for motor control.

A study which examined post-spike CV changes in human median axons found CV to decrease from 69 m/s to 60 m/s when two stimuli were given 0.8 ms

apart (Gilliatt and Willison 1963). If, then, CV can slow by as much as 15% in RRP or during the subexcitability period, and if it can increase by as much as 15% in the superexcitability period[4], then in humans this would impose as much as a 5 ms variation in the conduction latency of the second spike. This would be for motor axons with a CV of 70 m/s propagating over a 1 meter length. However, see Fig 7 in Bucher and Goillard (2011) for the effects of a spatial component to activity-dependent CV changes which would act to decrease this 5 ms variation in latency. More specifically, if during initial recruitment TA motor units often fire with a double discharge at a frequency around 100 to 300 Hz (*i.e.* an interval corresponding to superexcitability/supernormal CV – cf. sodium pentobarbital rats in Fig 4.8, Desmedt and Godaux 1977, Zajac and Young 1980, Gorassini *et al.* 2000) and if SOL motor units fire with a doublet at a lower frequency around 50 to 100 Hz (*i.e.* an interval corresponding to subexcitability/subnormal CV – cf. Fig 4.8, Gorassini *et al.* 2000) this could indeed decrease the latency of the second TA axon spike but increase the latency of the second SOL axon spike, producing effects on the "catch-like property" which are opposite in TA compared to the SOL muscle. The "catch-like property" of muscle fibers, whereby two consecutive motor unit discharges, separated by approximately 3 to 20 ms, increase the force of muscular contraction (see Thomas *et al.* 1999, Garland and Griffin 1999), could be facilitated in TA but inhibited in SOL. These effects on the catch-like property would be due simply to the pattern of CV fluctuation which occurs within RC. It barely needs mentioning that facilitation of the catch-like property by TA axons would match quite well with the general function of TA

motor units, which are typically fast and powerful units in most species. However, at the present time this is purely conjectural, and more direct investigations are required. In these directed studies the CV recovery cycles of TA and SOL axons would need to measured, in combination with the effects of axon discharge rates on TA and SOL muscle unit contractile properties.

5.3 Rheobase and SDTC Findings

Rheobase was significantly larger in fast compared to slow motor axons. As discussed in section 2.4.2.5, rheobase is influenced by a number of factors such as temperature, stimulus geometry, the target CMAP, and nodal axon properties (Bostock *et al.* 1983, Mogyoros *et al.* 1996, Bostock and Rothwell 1997). Since we stimulated TA and SOL axons from the same location (sciatic notch), and also since the temperature and target CMAP were strictly controlled in our study, it is likely that stimulus geometry is similar between TA and SOL axons. Most likely, the significant difference in rheobase between fast and slow motor axons reflects a difference in nodal axon properties. A lower rheobase is believed to be caused by larger levels of persistent Na^+ conductance (Bostock and Rothwell 1997), and so it is possible that slow motor axons have greater persistent Na^+ conductance. However, it is possible that other active axon properties (i.e. I_H) can also affect rheobase. There have been no additional studies which have substantiated the proposition by Bostock and Rothwell that rheobase is more strongly influenced by persistent Na^+ conductance than other axon properties. Indeed, a modeling study suggested that passive properties such as nodal width and axoplasmic resistivity

have a greater influence on axon rheobase than sodium conductance (Bostock 1983).

SDTC is thought to be positively correlated to the level of axon Na^+ conductance (Bostock and Rothwell 1997, Burke *et al.* 1998, Bostock *et al.* 1998, Krishnan *et al.* 2009). Since we found no difference in the SDTC of fast and slow motor axons, this may support the idea that Na^+ conductances are similar between these two axon groups. SDTC is believed to be influenced by persistent Na^+ conductance more than transient Na^+ conductance (Burke *et al.* 1998). It is unclear whether or not persistent Na^+ conductance arises from a unique channel or the same channel responsible for transient Na^+ conductance on axons (Krishnan *et al.* 2009), although it is apparent that persistent Na^+ conductance results from channels active near the resting membrane potential and that this current only accounts for ~2.5% of the total current produced by voltage-gated, TTX-sensitive Na^+ channels (Bostock and Rothwell 1997).

Although SDTC from our ketamine/xylazine rats suggests there is no difference in persistent Na^+ conductance between fast and slow motor axons, there are two other findings in this thesis which contradict this view. First, the finding that rheobase is significantly smaller in slow motor axons promotes the possibility of greater persistent Na^+ conductance in these axons. Second, in our sodium pentobarbital rats, SDTC in fast motor axons was 0.22 ± 0.02 ms while SDTC in slow motor axons was 0.32 ± 0.03 ms (mean ± SEM, n=5; compare with section 4.1.6 where the ketamine/xylazine rats were found to have a SDTC of 0.24 ± 0.01 ms and 0.28 ± 0.02 ms in TA and SOL axons, respectively). This is inconclusive,

however, and a follow-up study should be conducted in which AET is performed in a larger sample of rats.

5.4 Trends in Axon Excitability Testing Parameters

In section 2.4.4.1, the issue was raised that several AET parameters appear to be interdependent (i.e. co-vary) with each other. In our results there was a significant main effect in hyperpolarizing TE and I/V (plus an interaction effect). Not surprisingly, there was correspondence between these two measures such that increased average values in TEh corresponded with increased values in hyperpolarized I/V (*i.e.* in comparison with SOL axons, TA axons showed greater excitability in both TEh and I/V – see comparison *s* in table 2.1 and also examples in Mori *et al.* 2010, Trevillion *et al.* 2010, Nodera and Rutkove 2012). What about other *trends* (i.e. differences that did not reach statistical significance)? Were the data in this study consistent with data from the studies in table 2.1? For instance, our data from chapter 4 shows the following trends in the group A parameters: in comparison with fast axons, slow axons have an increased TEh overshoot, increased hyp I/V slope, decreased superexcitability, increased subexcitability (using the sodium pentobarbital data plotted in section 4.2.1), and an increased SDTC. The relationship amongst these parameters in table 2.1 was increased TEh -40% overshoot corresponding with an increased Hyp I/V slope as well as an increased superexcitability, increased subexcitability, and increased SDTC. Thus the trend in table 2.1 is very similar to the trend in our TA and SOL

axon data with the exception of superexcitability. There are three possible explanations for why the relationship of superexcitability to other parameters does not conform to the trend found in table 2.1:

(i) The magnitude of superexcitability is not different between TA and SOL axons, or

(ii) The trend found in table 2.1 arose by chance. Therefore this means that superexcitability and the other group A parameters vary independently of one another, or

(iii) We have specifically examined TA and SOL axons in this study because they generally represent axon properties of fast and slow motor units, respectively. By contrast, none of the studies in table 2.1 specifically compared axon properties of fast vs. slow motor units, or axon properties of highly active vs. less active motor units. Therefore, the data from each study in table 2.1 may have arose from averages across multiple motor unit types thereby blurring the differences between axons of fast motor units and slow motor units. The AET data trend in this thesis represents a different trend resulting from the unique differences between fast and slow motor units. It is well known that the slow motor units of SOL are activated much more often but at a much lower instantaneous frequency than the fast motor units of TA. None of the studies from table 2.1 have explicitly demonstrated that differences in activity patterns are contributing to the significant differences that they found. Thus, it is possible that when axons are categorized according to the schema, fast vs. slow motor unit type, a different pattern emerges from that seen in table 2.1. This new pattern shows that superexcitability is not interdependent with the other 7 group A parameters. Differences in activity may exert pressure on superexcitability to "adapt" uniquely. As mentioned above in section 5.2, it is possible that superexcitability in TA axons facilitates contractile properties of the TA muscle. Moreover, the Bonferoni post-hoc tests showed that TA axon excitability is significantly greater than SOL axon excitability at 3.2 ms and 4.0 ms in RC – these are interspike intervals which precede peak superexcitability by only a couple milliseconds (peak superexcitability typically occurs between 5 ms and 8 ms) and which match well with the initial doublet firing frequencies of TA motor units (see section 5.2). In addition, the sodium pentobarbital rats showed that at a delay of 6.3 ms the average superexcitability in TA axons was over three times larger than the average superexcitability in SOL axons in RC (see section 4.2.1). This gives some evidence that superexcitability is indeed different between TA and SOL axons.

We favour the last possibility over the first two listed above, as we have demonstrated that in the ketamine/xylazine (and probably also the sodium pentobarbital rats) TA and SOL axon excitability are significantly different at early delays in RC. In section 4.1.5 we showed that ketamine/xylazine rats did not exhibit any superexcitability. On the other hand, superexcitability is normally present in multiple species and in a multitude of different axons from different muscles, which is attested to by a number of AET studies in the literature. Importantly, our sodium pentobarbital rats showed average superexcitability at 6.3 ms delay to be three times larger in TA axons compared to SOL axons. It is plausible that if we had a larger sample size superexcitability would have been found to be significantly larger than SOL axon superexcitability. The superexcitability period in most axons is a relatively brief interval that begins as early as 3 ms and ends as late as 15 ms immediately after a single action potential, and it is possible that this feature is not just an artifact but instead has a functional role in the axon which may augment overall MN and motor unit functioning. The unique plasticity of superexcitability in response to different activity patterns would make sense under this hypothetical framework. It also may explain why the interdependence between superexcitability and other group A parameters, seen in table 2.1 (wherein none of the studies explicitly demonstrated that they were comparing axons with different activity patterns), is conspicuously absent in our study of TA and SOL axons. This is despite our results showing that the other 7 group A parameters retained the same relationships that were seen in table 2.1. In summary, our findings are not incongruous with the possibility that there is

interdependence amongst group A parameters, as discussed in section 2.4.6.1. It is possible that superexcitability may be uniquely sensitive to different activity patterns compared to the other group A parameters.

5.5 Implications for Understanding the Motoneuron and Motor Unit

We have found experimental evidence that the activity of hyperpolarization-activated inwardly rectifying conductance (I_H) differs significantly between TA and SOL axons. First, from our findings, we see that the physiology of axons in fast motor units differs significantly from the physiology of axons in slow motor units. This supports previous comments in editorials related to axon physiology, which discussed the idea that peripheral axons adapt to their activity patterns (Burke 2007, Kuwabara 2009). But what is/are the guiding force(s) behind these adaptations? It seems unlikely that energy conservation is a significant contributing factor in regard to I_H adaptations. Since slow motor axons have greater daily activity they likely utilize greater overall amounts of energy than fast motor axons. And from this study we have found that slow motor axons, as represented by SOL axons, have greater I_H activity which probably either increases energy expenditure in the cell or else does not significantly affect it (see section 5.1). Therefore, in slow motor axons increased I_H would not be caused by attempts at energy conservation in the cell. In addition, the function of somas and muscle fibers of fast motor units is, in part, to facilitate strong and rapid movements. It would therefore run counter to the adaptations seen in adjacent

compartments if adaptations in fast motor axons had energy conservation as a primary goal. Rather, the properties of somas and muscle fibers in fast and slow motor units are often coordinated in such a way that facilitates function but which does not necessarily facilitate energy conservation (cf. section 2.5; see also Henneman and Olson 1965, Zengel *et al.* 1985, Kernell 1992), and likewise it seems possible that axon properties are coordinated in such a way which enhances the function of the neuromuscular system but which does not make energy conservation a top priority.

It can be hypothesized, then, that adaptations in axon I_H confer a functional advantage to the cell, just as the adaptations in the compartments and cells adjacent to the axon confer functional advantages. The simplest explanation for our findings is that SOL axons experience greater amounts of activity-dependent hyperpolarization than TA axons thereby leading to a greater need of I_H in SOL axons. Perhaps the effects of axon hyperpolarization in slow motor axons, if left unopposed by I_H, could cause hyperpolarization at the MN soma and thus directly influence motor unit activity. The increased I_H in slow motor axons may help to counteract hyperpolarization caused by the activation of slow K^+ conductance and the electrogenic Na^+/K^+ pump after high-frequency firing. On the other hand, *decreased* activity of I_H in fast motor axons may allow for a slightly hyperpolarized membrane potential which in turn may facilitate superexcitability or have other effects on RC which may assist in the overall function of fast motor units. Various other conjectures could be made as to the physiological relevance of differential I_H expression in fast and slow motor axons. However, it is

impossible to give a full account of the influences of I_H in motor axons due to the paucity of knowledge of the HCN channels in motor axons. HCN channels are known to be greatly influenced by different intracellular and extracellular modulators (Robinson and Siegelbaum 2003, Biel *et al.* 2009), but the extent of modification of these channels by ligands inside the peripheral axon proper is virtually unknown.

The findings in this study serve to highlight the importance of the axon as something more than a mere conduit of digital information (Debanne *et al.* 2011, Bucher and Goaillard 2011). Moreover, this study and evidence from table 2.1 strengthens the hypothesis that motor axon physiology has implications which go beyond the "faithful transmission of an all-or-nothing signal", since a wide variety of nerves and axon sub-groups display a unique set of AET properties despite having no obvious differences in CV (see table 2.1). It seems that we should no longer look to CV as the most important bioelectric property in motor axons, but rather there should be an increased focus on the multiple underlying ionic conductances which influence CV as well as other motor axon bioelectric properties. In addition, these properties may prove to be more plastic and may respond more consistently to various chronic activity patterns compared to CV. Also, it is possible that there is coordination amongst motor axon bioelectric properties with implications that are unknown. This exploration of the relationship between motor axon physiology and motor function is in its infancy, however, and to our knowledge there are presently no studies which have simultaneously analyzed multiple axon bioelectric properties along with their

motor unit and muscle fiber properties. Additional investigation of the function of I_H and HCN channels along the axon would serve an important role in the quest to better understand the influence of motor axon properties on whole motor unit behavior and motor control.

5.6 Implications for ALS Research

In ALS, there is a selective atrophy of fast over slow motor units (Dengler *et al.* 1990, Frey *et al.* 2000, Hegedus *et al.* 2008). We have shown via AET that fast and slow motor axons differ in one or more ionic conductances. This foreshadows the possibility that some differences in AET between healthy controls and ALS patients are non-pathological in their origin. To date, there have been two recent AET studies which have looked at how the degree of motor impairment can affect AET in ALS patients (Kanai *et al.* 2006, Chea *et al.* 2012). The findings from these two studies indicate that axon excitability is different between ALS patients with little impairment (i.e. peak CMAP is greater than 5 mV) and those with larger impairment (i.e. peak CMAP is less than 1 mV) of motor function. In total, there have been eight studies which have statistically analyzed multiple AET parameters in ALS patients compared to healthy controls (Mogyoros *et al.* 1998a, Mogyoros *et al.* 1998b, Vucic and Kiernan 2006, Kanai *et al.* 2006, Vucic *et al.* 2007, Nakata *et al.* 2009, Vucic and Kiernan 2010, Cheah *et al.* 2012), and it is apparent that in ALS there are complex changes in multiple AET parameters which are dependent on the stage of disease progression. It is possible that changes to the relative proportions of fast and slow motor axons can

partly account for some of the AET changes.

In light of the differences between fast and slow motor axons found in this thesis, the AET study with the most pertinent finding comes from Kanai *et al.* (2006). In the study by Kanai and colleagues, ALS patients were divided into three sub-groups after the CMAP from abductor pollicus brevis, produced through stimulation of the median nerve at the wrist, was compared to normative values from healthy controls: (a) preserved CMAP (greater than 5 mV), (b) moderate CMAP reduction (CMAP between 1 and 5 mV), and (c) severe CMAP reduction (CMAP less than 1 mV). It was found that the ALS patients with the severe CMAP reduction (i.e. patients in a more advanced stage of the disease) had significantly larger excitability than healthy controls in TEh -40% (100-110 ms). This difference was not observed in the other two ALS sub-groups. This is consistent with the idea that ALS patients with the most severely reduced CMAP have had a relative increase in the number of slow motor axons, and this has increased values for the measure "TEh -40% (100-110 ms)".

In other AET parameters, the trends seen across the ALS patient sub-groups are more complex. Superexcitability is increased in patients with preserved CMAPs, is increased even more in patients with moderate CMAP reduction, but is normal in patients with severe CMAP reduction, in comparison with healthy controls (Kanai *et al.* 2006). This finding for superexcitability has been substantiated in a more recent study which has measured the changes in different ALS patients sub-groups at the beginning and end of a 12-week period. In ALS patients with a preserved peak CMAP (less than a 10% reduction in peak

CMAP), superexcitability significantly increased after 12 weeks (Cheah *et al.* 2012). When patients were divided into two groups on the basis of whether there were changes to fine motor skills, superexcitability decreased after 12 weeks in the group which demonstrated loss of fine motor skills (Cheah *et al.* 2012).

These distinct changes to superexcitability in ALS patients with preserved motor function vs. impairment of motor function are consistent with the following idea: axon pathology in ALS generally causes superexcitability to increase (see Kanai *et al.* 2006, Vucic and Kiernan 2006, Vucic and Kiernan 2010, Cheah *et al.* 2012); however, patients with large motor impairment probably have a large increase in the proportion of slow motor axons within their nerves. This increase in the proportion of slow motor axons then "opposes" these pathological changes to superexcitability and, when the influence of the change in proportion is great enough, causes a decrease in superexcitability (Kanai *et al.* 2006, Chea *et al.* 2012). This is because slow motor axons probably have smaller superexcitability than fast motor axons. Although we found significantly greater excitability in fast vs. slow motor axons at delays just prior to superexcitability in RC in the ketamine/xylazine rats of this thesis (at 3.2 and 4.0 ms delays), we did not actually find superexcitability - where threshold drops below baseline - in either of the axon groups. The lack of superexcitability found in this thesis is possibly due to the effects of ketamine/xylazine. However, in the RC of sodium pentobarbital rats, there was evidence that fast motor axons have greater superexcitability than fast motor axons (i.e. at a delay of 6.3 ms the average threshold increase was -9.36% in fast compared to -2.74% in slow motor axons),

although we did not perform any statistical analyses on the sodium pentobarbital rats due to the small sample size.

Finally, when rheobase is compared amongst ALS patient sub-groups separated on the basis of their CMAP impairment, we see additional evidence that a change in the proportion of fast vs. slow motor axons confounds AET results. When patients with more than a 10% reduction in peak CMAP are compared to patients with less than a 10% reduction in peak CMAP after a 12-week period, rheobase is found to be significantly less in the former patient sub-group (Cheah *et al.* 2012)[6]. In this thesis, we found that slow motor axons have a significantly lower rheobase than fast motor axons. Therefore, this difference in rheobase is consistent with the idea that in ALS patients with greater motor impairment, the relative increase in the proportion of slow motor axons causes AET measures to shift towards values that reflect slow motor axon properties. To a certain degree, changes in some AET measures in ALS patients at a later stage in the disease reflect non-pathological changes. These non-pathological changes are caused by an alteration in the proportion of fast vs. slow motor axons.

It must be mentioned that the trend in SDTC seen in the three ALS patient sub-groups from Kanai *et al.* (2006) is not completely consistent with the idea that greater levels of motor impairment should cause AET measures to shift towards values possessed by slow motor axons. SDTC has been shown to be significantly greater in ALS patients than healthy controls, in multiple AET studies which did not subdivide ALS patients according to their level of motor impairment (Mogyoros *et al.* 1998a, Vucic and Kiernan 2006, Vucic *et al.* 2007, Vucic and

Kiernan 2010). The findings from this thesis indicate that SDTC may be larger in slow compared to fast motor axons (where $p = 0.07$ from paired t-tests in ketamine/xylazine rats; however, average SDTC changed from 0.28 to 0.32 ms in slow motor axons but from 0.24 to 0.22 ms in fast motor axons when changing from ketamine/xylazine to sodium pentobarbital data). Therefore, we might expect that SDTC is the largest in patients with the largest motor impairment. However, average SDTC was not larger in ALS patients with great compared to little CMAP changes (Kanai *et al.* 2006). When comparing ALS patients and healthy controls, SDTC was significantly larger in patients with a preserved CMAP (Kanai *et al.* 2006). In contrast, SDTC was not significantly different between patients with a severe CMAP reduction and healthy controls (Kanai *et al.* 2006). The reason for this is unclear, but it is possible that these trends in SDTC amongst ALS patients with different motor impairment levels reflect a tension between pathological vs. non-pathological influences on SDTC. From Table I in Kanai *et al.* (2006), it is apparent that the small sample size (and the corresponding large SEM) from the patients with a severe CMAP reduction may have caused a false negative finding (i.e. a Type II statistical error) in the comparison between patients with a severe CMAP reduction and healthy controls. This may indicate that SDTC is not actually different across patients with varying levels of motor impairment. It is possible that when a larger sample size is obtained, SDTC may be found to be significantly larger in ALS patients with lower levels of impairment.

The changes to AET measures which have been observed in ALS patients

are complex. If it were possible to determine the relative effects of slow and fast motor axons in each of the AET measures, while also accounting for the effects of collateral re-innervation in ALS, then AET would be much more accurate in determining the mechanisms of axon pathophysiology in ALS. Aside from SDTC, it seems that an increase in the proportion of slow motor axons does, at least partly, account for the observations in ALS patients with large motor impairment (Kanai *et al.* 2006, Cheah *et al.* 2012).

5.7 The Effects of Ketamine/Xylazine vs. Sodium Pentobarbital

The statistical analyses which compared AET responses to ketamine/xylazine vs. sodium pentobarbital (section 4.2) were not planned in this thesis. Rather, they were performed after the serendipitous finding that these anaesthetics produce markedly different responses in multiple AET parameters. Therefore, although we did not investigate the anaesthetic differences to the same extent that we investigated TA vs. SOL axons, we nevertheless think it useful to remark on a few observations. First, from our results in section 4.2.1 it can be seen that there are statistically significant differences in specific early delays in RC. Second, in section 4.2.2 we see there are multiple statistically significant differences in TE between rats given sodium pentobarbital compared to rats given ketamine/xylazine. In regard to TEh, the rats given ketamine/xylazine had significantly less threshold increases *only at early delays*. Note that later delays in TE (those which are later than 10-20 ms after the onset of the polarizing pulse)

did not produce any significant differences between the anaesthetics, in both TEd and TEh. Taken together, these significant differences in TE, along with *the lack of* any significant differences in I/V (where measurements are made at a delay of 200 ms compared to a delay of 10-20 ms for the early delays in TE), this provides some evidence that an ionic conductance with fast kinetics (with a time constant of activation possibly as small as 20 ms) is altered by one or both of the anaesthetics used in this thesis. In other words, due to the differential AET responses in rats given ketamine/xylazine compared to rats given sodium pentobarbital, we have evidence that at least one of these anaesthetics alters certain AET measurements at early delays.

In line with the recognition that axons often process complicated information beyond that of all-or-none signals, in recent years there has been mounting evidence, in various tissues, of direct modulation of ionic conductances along the axon proper by ligands and/or neuromodulators. We will not appraise the evidence here (see Bucher and Goaillard 2011, Debanne *et al.* 2011, Stys 2011), but will instead focus only on the possible reasons for why motor axons might respond to the anaesthetics used in this study, ketamine/xylazine and/or sodium pentobarbital. The only ion channel through which sodium pentobarbital is known to exert its anaesthetic actions is through $GABA_A$. In peripheral sensory axons, the main effect of sodium pentobarbital via $GABA_A$ appears to be depolarization of the axon membrane potential (there is a depolarizing instead of a stereotypical hyperpolarizing action of $GABA_A$ most likely due to a higher concentration of chloride in the axon compared to other neuronal compartments -

Bhisitkul *et al.* 1987, Tomsic and Bajrovic 2000, Bucher and Goillard 2011). Although the presence of $GABA_A$ receptors has been documented in myelinated sensory axons, the presence of these receptors on motor axons appears to be insignificant (Bhisitkul *et al.* 1987).

We consider it unlikely that sodium pentobarbital is affecting any AET measurements in TA or SOL axons for three reasons: (i) It appears there is not physiologically significant levels of $GABA_A$ receptors on motor axons, as discussed above. (ii) When comparing the RC waveforms in sodium pentobarbital rats vs. ketamine/xylazine rats (discussed in section 4.2.1 and displayed in Fig 4.7) we can plainly see that the averaged RC waveform from sodium pentobarbital rats is much more similar to the RC waveform from rodent studies with various anaesthetics and *in vivo* human studies where no anaesthetics were used (*i.e.* compared to human studies, the timing of the RRP, superexcitability, and subexcitability is quite comparable in the sodium pentobarbital rats whereas these properties are highly unusual in ketamine/xylazine rats. Also, the magnitude of superexcitability and subexcitability is similar between sodium pentobarbital rats and human studies, whereas the ketamine/xylazine rats give a relatively small superexcitability and a greatly diminished subexcitability. See Schwarz *et al.* 2006, George and Bostock 2007, and Mori *et al.* 2010 for published rat RC examples and Boerio *et al.* 2009 for a published mouse example. See also Kiernan *et al.* 1996, Krishnan *et al.* 2004, Bae *et al.* 2009, and Jankelowitz and Burke 2009 for RC examples of axons innervating various human muscles). (iii) Qualitatively speaking, TE in humans is much more similar

to TE in the sodium pentobarbital rats than TE in the ketamine/xylazine rats (*i.e.* the waveform for delays across phases S1 and S2 in TEd +40% are normal in the sodium pentobarbital rats whereas in ketamine/xylazine rats the waveform at these delays looks unusual, in both TA and SOL axons).

As indicated above, there are marked irregularities in several AET parameters in the ketamine/xylazine rats. We will focus on abnormalities in TE and RC as these data were included in section 4.2. Before delving into the possible ionic mechanisms underlying our AET results in section 4.2, we shall first note that the multiple significant differences between ketamine/xylazine and sodium pentobarbital rats in TEh (at early delays) strongly indicates that, at the very least, I_H is affected by ketamine/xylazine. It is possible, however, that other ionic conductances besides I_H are also affected by the anaesthetics. In addition to TE, RC also contained significant differences between ketamine/xylazine and sodium pentobarbital rats in this study. Since measurements in RC are traditionally thought to be unaffected by I_H, this may provide evidence that multiple ion conductances are altered by one or both drugs used in the ketamine/xylazine cocktail.

There are a number of extrasynaptic receptors on motor axons which could possibly account for the AET irregularities seen in the ketamine/xylazine rats. Ketamine is well known for its antagonism of NMDA receptors on DRG neurons and along peripheral sensory axons (Sato *et al.* 1993, Coggeshall and Carlton 1998, Carlton 2009, Sigtermans *et al.* 2009). There is also speculation as to whether or not ketamine modulates multiple receptors in addition to NMDA

receptors. There is some evidence that ketamine is a D(2) dopamine receptor agonist, a serotonin 5-HT2 agonist, a substance P receptor antagonist, a sigma receptor antagonist, and a nicotinic acetylcholine receptor antagonist (Scheller *et al.* 1996, Wagner *et al.* 2001, Kapur and Seeman 2002, Okamoto *et al.* 2003, Seeman *et al.* 2009), although the evidence is limited as it is mostly from *in vitro* experiments and/or the role of these receptors in motor axons is unclear. Of more direct relevance to motor axons, ketamine has been shown to directly alter voltage-gated Na^+ channels, and possibly K^+ channels as well, although the mechanism by which this occurs is poorly understood. It appears that voltage-gated Na^+ channels have an overall decrease in conductance plus a rightward shift in the voltage curve of activation in the presence of ketamine, with the minimum ketamine concentrations required to alter channel properties ranging from 0.01 mM to 2.0 mM depending on species type and the experimental preparation (White *et al.* 1980, Arhem and Rydqvist 1986, Frenkel and Urban 1992, Brau *et al.* 1997, Zhou and Zhao 2000)[6]. The normal plasma concentrations of ketamine found in general anaesthesia in humans are perhaps just at the threshold at which ketamine may effect voltage-gated Na^+ channels and possibly K^+ channels as well (in one study plasma concentration peaked at 0.02 mM following administration of 8.5 mg/Kg ketamine in humans, although normally plasma concentrations do not exceed 0.01 mM – from White *et al.* 1980 and Idvall *et al.* 1979). Our study administered 60 mg/Kg ketamine to induce general anaesthesia in rats and therefore it is possible that rat plasma concentrations approached or exceeded the concentration required for alteration of voltage-gated Na^+ channel properties.

5.7.1 Were Voltage-gated Na^+ and K^+ Channels Influenced by Ketamine/Xylazine?

In comparison with AET studies which have manipulated Na^+ or K^+ conductances in order to investigate the effects of these channels on AET measurements, the results from our ketamine/xylazine rats are quite unique. There are several studies which have measured AET in subjects with modified Na^+ conductances, by using mexiletine to block Na^+ channels in neuropathic patients or by performing AET in an individual with acute TTX poisoning. In addition, the role of Na^+ in AET has been investigated by using genetically altered mice with mutations in the SCN1B gene or SCN8A gene, which are believed to alter transient Na^+ and possibly persistent Na^+ conductances as well. In all of the subjects which had manipulated Na^+ channels, it was found that there was either no effect on TEh (seen when mexiletine is applied or the SCN8A gene is mutated – Kuwabara *et al.* 2005, Isose *et al.* 2010, Sittl *et al.* 2011) or else an excitability *decrease* in TEh (seen after TTX ingestion and in mutation of the SCN1B gene – Kiernan *et al.* 2005a, Kiernan *et al.* 2005b), compared to normal controls. These findings are opposite to those in the present study, where rats given ketamine/xylazine had significantly *greater* excitability at early delays in hyperpolarizing TE in comparison with the sodium pentobarbital rats (see section 4.2.2). In addition, dysfunction of Na^+ conductances in the above studies either produced no change in the RRP or else a significant *decrease* in the RRP, a finding also in contradiction to our present results for the ketamine/xylazine rats (ketamine/xylazine rats can be seen to have a greatly exaggerated RRP – see section 4.2.1). Thus, under the assumption that ketamine/xylazine has a significant effect on motor axon physiology while

sodium pentobarbital does not, the idea of ketamine/xylazine disrupting only Na^+ conductances (fast and/or persistent Na^+ conductance) is an inadequate account of our present results.

Impairment of fast K^+ conductance by ketamine/xylazine cannot fully account for our present results either. In a study which examined patients suffering from episodic ataxia type 1 due to a mutation in the gene encoding kv1.1 (a subunit of the ion channel responsible for the fast K^+ conductance), there is an increase in excitability at early delays in TEd, a decrease in excitability across late delays in TEh, an increase in superexcitability and subexcitability in RC, and a leftward shift in subexcitability (Tomlinson *et al.* 2010). 4-AP, a blocker of fast K^+ channels, produces changes similar to those caused by the kv1.1 mutation: 4-AP increases and prolongs after-depolarization after an action potential (analogous to superexcitability) and also increases AHP (analogous to subexcitability- from Baker *et al.* 1987). 4-AP also produces an increase in excitability across all delays in TEd plus a decrease in excitability in TEh (Yang *et al.* 2000). Consequently, it appears that antagonism of the fast K^+ conductance creates changes in AET measures which are opposite to the changes seen in the ketamine/xylazine rats. It is therefore possible that ketamine/xylazine is acting as an *agonist* of the ion channel underlying fast K^+ conductance. In line with this, it is interesting to note that there is significantly lesser excitability in patients with a Kv1.1 mutation compared to healthy controls only for the first 100 ms or so after a hyperpolarizing conditioning pulse, but at longer delays (*i.e.* 200 ms) there is no significant differences. This is a similar trend to what is found in our

ketamine/xylazine rats, where there are significant abnormalities only for the first 20-30 ms after the hyperpolarizing conditioning pulses in TE, but at longer delays in TE (as well as in I/V) there are no significant differences. On the other hand, there are two arguments against the idea that ketamine/xylazine is an agonist of fast K^+ channels: (i) a previous report showed ketamine to be an antagonist of K^+ channels (Arhem and Rydqvist 1986). (ii) In the ketamine/xylazine rats there is an increase in excitability only at the 20-30 ms delay in TEh, whereas in patients with a Kv1.1 mutation there is a significant abnormality in excitability at the 100-109 ms delay (Tomlinson *et al.* 2010).

Impairment of slow K^+ conductances by ketamine/xylazine also cannot fully explain our present results. In an AET study which treated rat sural nerves with flupirtine, an agonist of Kv7 (the ion channel underlying the slow K^+ conductance), there was a significantly decreased refractoriness as well as a significantly increased superexcitability and subexcitability in comparison to controls (Sittl *et al.* 2010). A pattern of changes opposite to those of flupirtine is consistent with the changes we see in our ketamine/xylazine rats (where increased refractoriness, decreased superexcitability, and decreased subexcitability are observed). However, since changes in other AET parameters were not reported by Sittl and colleagues (presumably because no significant differences were observed), flupirtine provides underwhelming evidence that the pattern of AET changes in our ketamine/xylazine rats is due to antagonism of Kv7 channels. Moreover, when XE991 (an antagonist of kv7 channels) is administered to rats, a pattern emerges which is in contradiction to the pattern produced by flupirtine and

which is also in contradiction to the pattern in our ketamine/xylazine rats (in other words, XE991 does not produce changes in AET which are opposite to the changes induced by flupirtine). Upon XE991 treatment, there is an increase in excitability across all delays in depolarizing TE, and there is no change in hyperpolarizing TE as well as very little qualitative change in the RC waveform (Schwarz *et al.* 2006). Finally, upon administration of retigabine (an agonist of Kv7 channels), hyperpolarizing TE has a decrease in excitability across all delays, while superexcitability in RC undergoes a slight reduction and shifts rightward (Nodera *et al.* 2011). A pattern of AET changes directly opposite to those produced by retigabine (*i.e.* TEh with an excitability increase across all delays in conjuction with superexcitability increasing and shifting leftward) is different than the pattern of changes we have observed in our ketamine/xylazine rats. In summary, there is one AET study using flupirtine which presents evidence that does not undermine the idea that ketamine acts as an antagonist of slow K^+ channels (Sittle *et al.* 2010), although another AET study which has used a different agonist of slow K^+ channels (Nodera *et al.* 2011) has produced different AET results which are in contradiction to the findings in the present study. In addition, another study which has used an antagonist of K^+ channels (Schwarz *et al.* 2006) reveals an entirely different pattern of changes in AET parameters which could not have been predicted by simply reversing the changes in AET induced by agonists of slow K^+ conductance. The study using XE991 as well as the retigabine study provide AET data which seems to argue against the idea of ketamine being an antagonist of slow K^+ conductance, although the flupirtine

study provides some evidence to the contrary.

Finally, there is a possibility that ketamine may be exerting its effects through NMDA receptors on the TA and SOL axons examined in this study. The presence of NMDA receptors on sensory axons is well documented as they may play a therapeutic role in the management of neuropathic pain (Carlton 2009). Since NMDA receptors in the periphery have been studied almost exclusively in sensory axons, however, there is a dearth of information regarding the possible presence of NMDA receptors on motor axons. One study which examined the distribution of NMDA receptors in the plantar nerves of rats found that over half of the examined axons contained these receptors (Coggeshall and Carlton 1998). Although the study above did not differentiate between motor and sensory axons when immunostaining NMDA receptors in the plantar nerve, the idea that motor axons contain NMDA is supported by fact that ventral MNs contain NMDA receptors (Petralia *et al.* 1994, Tolle *et al.* 1995, Popratiloff *et al.* 1996). Therefore, there is a possibility that ketamine may be acting via NMDA receptor inhibition to cause the statistically significant decreases in excitability in RC at early delays (see section 4.2.1) as well as statistically significant increases in excitability at early delays in TE (see section 4.2.2) in the present study. However, it is difficult to perceive exactly how NMDA receptors could mediate such changes in excitability (as well as membrane potential) in motor axons, especially when considering that the conductances which have been altered appear to have quite fast kinetics (*i.e.* a conductance with a time constant of activation possibly as short as 10 ms). Therefore, the possible role of NMDA

receptors in mediating changes to motor axon AET remains purely speculative, if not unlikely.

5.7.2 Summary and Possible Mechanisms

Significant differences exist between rats given sodium pentobarbital and rats given ketamine/xylazine, in a number of AET parameters: TEd at 20-30 ms (in TA axons only), TEh at 20-30 ms (TA and SOL axons), as well as a few early delays in RC (TA axons only). Given that AET measures from the sodium pentobarbital rats are qualitatively similar to the AET measures of humans and a variety of animal studies (see section 5.7.1), and also since $GABA_A$ receptors seem to play no physiological role on motor axons (Bhisitkul *et al.* 1987), it is unlikely that sodium pentobarbital is causing any significant changes to the bioelectric properties of TA and SOL axons. Rather, AET measures in the ketamine/xylazine rats are abnormal, and so it appears that this anaesthetic is changing axon bioelectric properties through modulation of ionic conductances in both TA and SOL axons. Ketamine is known to target a variety of receptors, although some of these receptors are unlikely to be present on motor axons (*i.e.* substance p receptors) and the presence of several of these receptors on peripheral motor axons has not been firmly established (such as 5-HT2, D(2) dopamine, and acetylcholine receptors). More importantly, ketamine is known to act as an antagonist of voltage-gated Na^+ channels and possibly also an antagonist of voltage-gated K^+ channels as well. We have cited a number of studies which have looked at the effect of voltage-gated Na^+ channels on AET parameters and have shown that it is unlikely that ketamine/xylazine alters AET parameters via

antagonism of Na^+ channels. Furthermore, we have provided evidence for why ketamine/xylazine is very unlikely to be an antagonist of voltage-gated fast K^+ channels (although we discuss above that *activation* of fast K^+ channels may produce AET results which are similar to the results from ketamine/xylazine rats in the present study). In addition, it is not likely that ketamine or xylazine exert effects on AET through antagonism of slow K^+ channels, although one study using flupirtine did provide some evidence to the contrary (Sittl *et al.* 2010). It is possible that ketamine is acting as an antagonist of NMDA receptors on TA and SOL axons. However, the presence of NMDA receptors on motor axons is yet to be determined (one study has raised the possibility of NMDA receptors being present on motor axons – see Coggeshall and Carlton 1998).

Although the potential targets of ketamine and/or xylazine discussed above are speculative and have little direct evidence, we have nevertheless shown by using AET data that this drug cocktail produces statistically significant increased excitability at 20-30 ms in TEh, but at larger delays these significant differences disappear. These differences are likely mediated by either (a) directly increased activation of I_H, or (b) decreased activation of other ionic conductances (*i.e.* slow K^+ current) which results in an overall decreased membrane conductance (see the "Discussion" section of Tomlinson *et al.* 2010 for this line of reasoning). Regardless of which of these two scenarios is correct, the conductance altered by ketamine/xylazine has fast kinetics (*i.e.* it exerts significant changes to axon accommodative properties after a hyperpolarizing pulse has been applied for only 20-30 ms), and therefore if ketamine/xylazine is

modulating HCN channels it would be via the HCN1 isoform (other HCN isoforms have time constants of activation which are at least 150 ms; see Robinson and Siegelbaum 2003, Biel *et al.* 2009). In summary, we can propose three potential mechanisms by which TE and RC are altered after administration of ketamine and xylazine in rats:

> (i) alteration of unknown receptors or ion channels (possibly 5-HT2, D(2) dopamine, acetylcholine, or other unknown receptors) on TA and SOL axons by ketamine and/or xylazine has resulted in a shift in the intracellular concentration of second messengers or ligands which modulate HCN1, ultimately causing a depolarizing shift in the voltage curves of activation for HCN1 channels, faster activation/inactivation of HCN1 channels, and/or a large increase in the permeability of HCN1 channels to cations. One or a mix of these alterations to HCN1 channels then renders significantly different TE values at early delays. The increased activation of I_H may also result in membrane depolarization which significantly alters the RC waveform (see Moalem-Taylor *et al.* 2007 for evidence of how cesium-sensitive I_H can effect RC. Also see Howells *et al.* 2012 where computer modeling evidence is given to support the hypothesis that increased I_H activation leads to depolarization of the resting membrane potential). In addition, modulation of the unknown axon receptors and/or the shift in the intracellular concentration of certain second messengers may also change the properties of axon ion channels which specifically alter RC.
>
> (ii) Antagonism of NMDA receptors on TA and SOL axons by ketamine alters axon excitability through unknown mechanisms. This ultimately manifests in an increased activation of I_H (possibly leading to membrane depolarization) and perhaps changes to other ionic conductances which selectively affect RC.
>
> (iii) Ketamine could possibly be an agonist of fast K^+ channels and an antagonist of slow K^+ channels. A disruption of this kind to these K^+ conductances would cause a complex change to axon excitability which apparently increases I_H (again, possibly leading to membrane depolarization). The selective agonism of fast K^+ conductances, however, would not be expected to produce the effects on RC that we have seen in the ketamine/xylazine rats (cf. Baker *et al.* 1987, Yang *et al.* 2000, Tomlinson *et al.* 2010). By the same token, antagonism of slow K^+ channels has not been shown to change RC in the way that we have observed in our ketamine/xylazine rats (cf. Schwarz *et al.* 2006). Therefore we consider this third potential mechanism, in which ketamine

activates fast K^+ channels and inhibits slow K^+ channels, less likely than the first two potential mechanisms.

5.8 Study Limitations

A limitation of this study was that the TA axons were assumed to be from fast motor units while SOL axons were assumed to be from slow motor units. Clearly, we would have been able to provide a more full account if we had simultaneously studied motor axon properties along with the mechanical properties of the innervated muscle fibers, or the electrophysiology of the adjacent soma. Had this been done, we could have better characterized the axons as coming from a 'slow' or 'fast' motor unit. Future studies performing AET on individual axons in combination with analysis of the parent soma or innervated muscle fibers, though technically difficult, would be beneficial since they would require less assumptions and would provide a more definitive characterization of axons from different motor units. In addition, these studies would allow a statistical investigation of the correlations between AET properties and muscle fiber or somatic properties. Furthermore, a discriminant analysis using AET measures from these studies, done in the same fashion as in Zengel *et al.* (1985), could find motor axon physiology highly useful for separating motor axons into the same three main “types” as their innervated muscle fibers.

A second limitation of this study was that we tracked on only one axon threshold level, $CMAP_{40\%}$. Previous AET studies have shown that motor axon physiology can change significantly according to the target threshold level of the

axons (Mori *et al.* 2010, Trevillion *et al.* 2010, Nodera and Rutkove 2012, etc.). If in the TA axon group we had tracked on multiple threshold levels and found there were no significant differences or correlations amongst these threshold levels, and then if we found the same to happen in SOL axons, yet still found TA axons to be significantly different than SOL axons at each threshold level, this would promote the appeal of distinguishing motor axons according to their motor unit type. Alternatively, there may be significant differences in AET data between threshold levels within TA or SOL axons, thereby underscoring the importance of axon threshold level. It is worth noting here that electrical stimulation is thought to recruit axons according to their location within a nerve more than according to their diameter (Doherty and Brown 1993, Major and Jones 2006). It is possible that in this thesis it was not motor unit type - and by extension differences in activity patterns - which were responsible for our statistically significant findings, but rather differences in the location of TA vs. SOL axons within the sciatic nerve[7]. However, it is difficult to perceive how differences in TA and SOL axon locations would have a systematic effect on AET, since inter-subject variability would presumably be very high if it were true. Nevertheless, a study investigating the influence of axon locations on AET would put this issue to rest. In addition, experimental investigation of the influence of axon diameter on AET is needed.

Finally, in section 5.7 we deliberated at length upon the evidence for why one or more anaesthetics used in this study may have influenced the AET data. After reviewing the relevant information regarding sodium pentobarbital and

ketamine/xylazine, as well as comparing the qualitative aspects of each of these datasets (*i.e.* the relative timing of superexcitability in RC), the evidence pointed to ketamine/xylazine altering both TA and SOL axon physiology. Specifically, unusual features in TE and RC were found in the ketamine/xylazine rats. Therefore, although there is no reason to suspect that ketamine/xylazine differentially influences TA and SOL axons, it is possible that our account of TA and SOL axon physiology is erroneous due to the effects of ketamine/xylazine. This does not seem very likely, however, as the trends in the sodium pentobarbital data were identical to the trends found in the ketamine/xylazine data. For example, when comparing TA vs. SOL axons in the sodium pentobarbital rats, the latter group had a tendency for SDTC to be larger, rheobase smaller, hyp I/V slope larger, less excitability at larger delays in TEh, and less excitability throughout RC. All of these trends are identical to those seen in the ketamine/xylazine dataset. Thus, in regard to the AET comparisons between TA and SOL axons, there is not a single trend found in the ketamine/xylazine rats which is different from the sodium pentobarbital rats.

5.9 Conclusion

The primary goal in this study was to compare the bioelectric properties of fast and slow motor axons. Multiple AET measures revealed that slow motor axons have greater activity of the hyperpolarization-activated inwardly rectifying conductance (I_H) than fast motor axons, endowing slow motor axons with a greater ability to resist hyperpolarization of the membrane potential. In addition,

the post-spike oscillation in excitability was found to be significantly different between fast and slow motor axons, with fast motor axons having greater excitability at early delays of 3.2 and 4.0 ms in RC. Together, these findings suggest that axon bioelectric properties adapt specifically and uniquely to the different activity patterns seen in fast and slow motor units. These adaptations are the result of changes to one or more ionic conductances, involving I_H at the very least. Had AET not been available for addressing our research question, it is highly likely that these important differences between fast and slow motor axons would have gone undetected by traditional nerve conduction measures such as CAP amplitude or CV.

We also found evidence that the anaesthetic cocktail, ketamine/xylazine, can substantially alter the results of AET. However, it was evident that both anaesthetics used in our thesis, ketamine/xylazine and sodium pentobarbital, showed identical trends amongst the various AET measures. Therefore, we do not suspect that the alteration of axon bioelectric properties by ketamine/xylazine confounded our results. This serendipitous finding from ketamine/xylazine supports previous observations that properties of the axon proper can be modified by ligands and/or neuromodulators (Bucher and Goaillard 2011, Debanne *et al.* 2011, Stys 2011). However, a more rigorous study with planned statistical comparisons is necessary to better elucidate the mechanisms by which anaesthetics such as ketamine/xylazine can alter peripheral axon properties.

Notes

1. It is perfectly reasonable to find greater "I_H activity" in a certain axon group using AET methodology while at the same time asserting that this same axon group has HCN channels with lower single-channel conductance (see Howells *et al.* 2012 where this is proposed for sensory vs. motor axons).

2. The experimental evidence for the idea that increased axon diameter results in a decreased superexcitability, advanced by McIntyre *et al.* 2001, is perhaps illuminated by experimental AET studies. Some articles have shown a trend for lower threshold axons to have a noticeably larger superexcitability (Nodera and Rutkove 2012) while other studies have reported no clear trend for superexcitability to vary according to axon threshold level (Kiernan *et al.* 1996, Trevillion *et al.* 2010, Mori *et al.* 2010). In regards to superexcitability, the inconsistency seen in the trends for axons of different thresholds means one or two things: (i) analyzing axons of progressively larger thresholds cannot be interpreted as analyzing axons of progressively decreasing axon sizes, and/or (ii) superexcitability is not influenced by axon diameter. Possibility (i) is supported by studies which have found evidence that electrical stimulation does not clearly recruit axons according to their diameter (Doherty and Brown 1993, Major and Jones 2006).

3. In these diseased states, TA and SOL axon RCs may be altered in different ways, which may or may not exacerbate the differences already present in their RC. Future studies which focused on the unique effect of the disease on the RC of specific groups of motor axons (*i.e.* axons from fast fatigable motor units) could provide a better understanding of aberrant motor functions.

4. There is a dearth of information regarding the change in CV during the superexcitability period. Bullock (1951) showed CV to increase by 10% in the RC of frog sciatic axons.

5. See Kanai *et al.* (2006), however, which found a different trend in rheobase changes across three patient sub-groups classified according to their peak CMAP.

When each of the three ALS patient sub-groups were compared to healthy controls, rheobase was not found to be significantly different. The different trend was possibly because this study divided patients using different CMAP criteria.

6. The second component of the cocktail anaesthetic, xylazine, is an α2 adrenergic agonist. α2 adrenoceptors are not known to play any functional role in peripheral motor axons, so it is unlikely these receptors are playing a significant role in the AET measures discussed in this thesis. However, it should be noted that recent evidence from dendrites in the prefrontal cortex has shown that α2 adrenergic agonism decreases cAMP availability which in turn decreases I_H activity (Wang *et al.* 2007). This supports the idea that it is only ketamine and not xylazine which has influenced the AET data, since we have found *increased* I_H activity in the ketamine/xylazine rats.

7. One may advance the hypothesis that the anatomical position of axons has a significant influence on AET data and therefore these may have contributed to the significant differences between TA and SOL axons in this study. Indeed, SDTC and rheobase are known to be influenced by the distance between the stimulating electrodes and the axons (Noble and Stein 1966, Bostock *et al.* 1983). However, in the measures which we have found statistically significant findings (TE, I/V, and RC), there is no evidence in the literature indicating that these factors, electrode distance to axons or anatomical location of axons, have any influence. In addition, if electrode distance to axons or anatomical locations of axons had a significant impact on AET measurements besides SDTC and rheobase, there would be such large inter-subject variability in these AET data that this would likely prevent significant differences from being detected between different axon groups (*i.e.* between median axons of different thresholds). This hypothesis is thus difficult to reconcile with the large number of significant differences found in table 2.1 amongst axons of different thresholds and amongst axons of different muscles.

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