Spike train propagation in the axon of a visual interneuron, the descending contralateral movement detector of *Locusta migratoria*

By

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Abstract

Neurons perform complex computations, communications and precise transmissions of information in the form of action potentials (APs). The high level of heterogeneity and complexity at all levels of organization within a neuron and the functional requirement of highly permeable cell membranes leave neurons exposed to damage when energy levels are insufficient for the active maintenance of ionic gradients. When energy is limiting the ionic gradient across a neuron’s cell membrane risks being dissipated which can have dire consequences. Other researchers have advocated “generalized channel arrest” and/or “spike arrest” as a means of reducing the neuronal permeability allowing neurons to adjust the demands placed on their electrogenic pumps to lower levels of energy supply. I investigated the consequences of hypoxia on the propagation of a train of APs down the length of a fast conducting axon capable of transmitting APs at very high frequencies. Under normoxic conditions I found that APs show conduction velocities and instantaneous frequencies nearly double that of neurons experiencing energy limiting hypoxic conditions. I show that hypoxia affects AP conduction differently for different lengths of axon and for APs of different instantaneous frequencies. Action potentials of high instantaneous frequency in branching lengths of axon within ganglia were delayed more significantly than those in non-branching lengths contained within the connective and fail preferentially in branching axon. I found that octopamine attenuates the effects of hypoxia on AP propagation for the branching length
of axon but has no effect on the non-branching length of axon. Additionally, for energetically stable cells, application of the anti-diabetic medication metformin or the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel blocker ZD7288 resulted in a reduced performance similar to that seen in neurons experiencing energetic stress. Furthermore both metformin and ZD7288 affect the shape of individual APs within an AP train as well as the original temporal sequence of the AP train, which encodes behaviourally relevant information. I propose that the reduced performance observed in an energetically compromised cell represents an adaptive mechanism employed by neurons in order to maintain the integrity of their highly heterogeneous and complex organization during periods of reduced energy supply.
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TABLE OF CONTENTS

ABSTRACT ........................................................................................................................ ii

ACKNOWLEDGEMENTS ...................................................................................................... iv

TABLE OF CONTENTS ..................................................................................................... v

LIST OF FIGURES AND ILLUSTRATIONS ................................................................ viii

LIST OF ABBREVIATIONS ............................................................................................... x

CHAPTER 1: INTRODUCTION ........................................................................................ 1

Hypoxia-tolerance ............................................................................................................. 2

The energetic cost of spike trains ................................................................................... 3

Cost/benefit analysis of spike trains in high performance cells ....................................... 7

Spike train propagation in a high performance visual interneuron .................................. 8

Hypoxia-tolerance in the locust ..................................................................................... 11

The AMP-activated protein kinase cascade; balancing supply with demand ................. 12

The role of hyperpolarization-activated cyclic nucleotide-gated channels in neuronal
performance under ideal and hypoxic conditions ...................................................... 13

Hypothesis ..................................................................................................................... 16

CHAPTER 2: MATERIALS AND METHODS ................................................................ 18

Animals and preparations .............................................................................................. 18

Evoked-stimulus and measured response ....................................................................... 22

Experimental procedure ............................................................................................... 24

Data analysis .................................................................................................................. 28
CHAPTER 3: RESULTS .................................................................................................. 35

Spike train propagation is compromised in hypoxic nerve cords ......................... 35

Effects of hypoxia on spike train propagation are not strictly uniform along the entire
length of DCMD .......................................................................................................... 43

Octopamine attenuates the effects of hypoxia in the ventral nerve cord on spike train
propagation .................................................................................................................. 44

Bath applications of metformin or ZD7288 compromise spike train propagation in
preparations with normoxic nerve cords ..................................................................... 46

Metformin and ZD7288 affect the amplitude, duration and shape of action potentials
as interpreted from their triphasic waveforms recorded extracellularly ....................... 52

Non-uniform additive increases in conduction delay over the entire length of DCMD
axon are significant in terms of arrival time and may result in the corruption of
temporally encoded information .................................................................................. 57

CHAPTER 4: DISCUSSION ............................................................................................ 64

Axonal conduction velocities observed under normoxic conditions are not supported
by DCMD during hypoxia ........................................................................................... 67

DCMD axon fails to support the propagation of high frequency action potentials
during hypoxia: Consequences for temporal coding ............................................... 73

Reduced DCMD performance during hypoxia: a trade off resulting in maladaptive
behaviour or an inherently adaptive strategy? ............................................................. 81

Mechanism of metformin action on DCMD axon ....................................................... 85

Conclusion: The virtues of DCMD as a model system ............................................. 87
LIST OF FIGURES AND ILLUSTRATIONS

Figure 1. Experimental overview ...................................................................................... 19
Figure 2. Normoxic and hypoxic nerve cord preparations .................................................. 21
Figure 3. Two-dimensional representations of the looming stimulus ............................... 23
Figure 4. Visualization of the scale of the DCMD axon relative to the connective ........ 25
Figure 5. Experimental/pharmacological time course ...................................................... 26
Figure 6. Information available from triphasic action potentials ...................................... 29
Figure 7. Individual responses to loom recorded from normoxic, hypoxic and hypoxic OA treated nerve cords ..................................................................................................... 35
Figure 8. Octopamine attenuates the time dependent hypoxia induced rundown of DCMD axonal conduction velocity for the length of axon contained within the mesothoracic ganglion ............................................................................................................................. 37
Figure 9. Spike train fidelity in the DCMD axon was compromised within the mesothoracic ganglion of hypoxic nerve cords ................................................................. 40
Figure 10. The effect of bath applied octopamine on the spike count and peak frequency of spike trains recorded from the axon of DCMD in hypoxic nerve cords ..... 41
Figure 11. Individual responses to loom during the bath application and washout of metformin and ZD7288; conduction delays plotted against their respective instantaneous frequency ........................................................................................................................ 46
Figure 12. Conduction velocity in DCMD axon was reversibly decreased by pharmacological manipulations with metformin and Z7288 in normoxic nerve cords .... 48
Figure 13. Peak frequency in the DCMD axon was reversibly decreased by pharmacological manipulations with metformin or ZD7288 in normoxic nerve cords and spike count was unaffected .......................................................................................................................................................... 50

Figure 14. Overlays of the triphasic waveforms for action potentials generated before during and after pharmacological manipulation ........................................................................................................ 52

Figure 15. Pharmacological manipulations of duration and amplitude during the second and third phases of the triphasic waveform ........................................................................................................ 53

Figure 16. Metformin reversibly affects the amplitude of the first phase in the triphasic waveform ............................................................................................................................................... 55

Figure 17. 2-D raster plots of all the APs within a spike train show that the triphasic waveforms for APs of various instantaneous frequencies are affected by pharmacological manipulations with either metformin or ZD7288. ........................................................................................................ 57

Figure 18. Multi-electrode triphasic action potential overlays .......................................................................................................................... 58

Figure 19. Interpreting the cumulative delay in DCMD axon incurred between the LGMD (source) and the FETi (destination) ..................................................................................................................... 60

Figure 20. Pharmacological manipulation of interspike-intervals ....................................................................................................................... 62
LIST OF ABBREVIATIONS

AMPK, AMP-activated protein kinase
ANOVA, analysis of variance
AP(s), action potential(s)
CNBD, cyclic nucleotide binding domain
DCMD, descending contralateral movement detector
FETi, fast extensor tibiae
HCN, hyperpolarization-activated cyclic nucleotide-gated
$I_h$, hyperpolarization-activated current
ISI, interspike-interval
LGMD, lobula giant movement detector
MMRCD, mean maximum relative conduction delay
MPFAPs, mean peak frequency of action potentials
MRCD, mean relative conduction delay
OA, Octopamine
PDE, phosphodiesterase
TTX, tetrodotoxin
CHAPTER 1: INTRODUCTION

Neurons require selectively permeable membranes as a prerequisite for the generation, propagation, and integration of electrical signals. It is, however, this same permeability that makes neurons highly susceptible to damage in the absence of oxygen. For the majority of animals their neurons rely on a constant supply of oxygen for the active maintenance of ionic gradients across their cell membranes. Electrogenic pumps move charged particles against their thermodynamic equilibrium, this work requires energy in the form of ATP and the amount required is positively correlated with membrane permeability. The Na\(^+/\)K\(^+\) ATPase is the main electrogenic pump involved in the maintenance of ionic gradients. Without sufficient energy to run the Na\(^+/\)K\(^+\) ATPase, neurons will eventually depolarize, resulting in the uncontrolled influx of Ca\(^{2+}\) through voltage-gated Ca\(^{2+}\) channels. This rise in intracellular calcium activates Ca\(^{2+}\)-dependent phosphatases and proteases accelerating the process of membrane depolarization, which eventually results in cell swelling and finally cell death (Hochachka 1986). Unlike mammals, hypoxia-induced membrane destabilizations of this kind are either slow to develop or non-existent in several of the lower vertebrates, neonates and diving animals as a direct result of the adaptive decrease in membrane permeability (Boutilier 2001). Without adequate amounts of oxygen the main protective measure for the maintenance of gradients is a reduction in membrane permeability. However, owing to the nature of
signal generation and propagation in neurons, a reduced permeability comes at the cost of reduced performance in neuronal signaling.

*Locusta migratoria* can survive in an atmosphere of 0% oxygen for more than 4 hrs, and have been shown to reversibly depress their metabolic rate in response to graded hypoxia with no significant effect on behaviour (Wegener 1995). Using the descending contralateral movement detector (DCMD) neuron of *L. migratoria* as a model, it is the aim of this thesis to characterize the reduced performance of this visual interneuron in response to a graded hypoxia as an indication of an adaptive reduction in membrane permeability, with an added attempt to identify pathways and targets involved in mediating performance.

**Hypoxia-Tolerance**

Studies of hypoxia-tolerant vertebrate species have shown that the natural defense strategy providing the greatest protection against hypoxia is an orchestrated suppression of energy turnover (Hochachka *et al.* 1996). In fact, one of the key differences between hypoxia-tolerant species and hypoxia-intolerant species is their relative abilities to reduce the demand that the electrogenic Na⁺/K⁺ ATPase places on a limited ATP supply during times of low oxygen (Boutilier 2001). In the case of a hypoxia-intolerant species, oxygen-limiting environments cause reduced activity of the Na⁺/K⁺ ATPase, and substantially reduced ATP concentration leading to continually depolarizing membrane. Neurons of a hypoxia-intolerant species in such an energetic state are in danger of
incurring damage as a result of the uncontrolled influx of extracellular and intracellular Ca\textsuperscript{2+} into the cytoplasm of the cell. In stark contrast, reduced activity of the Na\textsuperscript{+}/K\textsuperscript{+} pump in a hypoxia-tolerant species experiencing an oxygen-limiting environment indicates a reduction in the number of energy expending processes performed by the neuron. Reducing expenditures on the costly processes of signaling can stabilize both the membrane potential and the ATP concentration of a neuron during time of reduced energy supply. As a result the hypoxia-tolerant neuron is protected against Ca\textsuperscript{2+} influx.

It was suggested over a decade ago that the elimination of a significant number of energy-requiring, function-related processes of a neuron could possibly act to protect it during times when its capacity to generate energy had been compromised (Ames et al. 1995). However, a number of problems previously identified (Hochachka et al. 1996) remain to be fully explained (i) how do cells/tissues “know” to turn on their hypoxia defense mechanism, (ii) exactly how is the ATP demand and supply down-regulated and to what extent and (iii) how are membrane electrochemical gradients stabilized.

*The energetic cost of spike trains*

Attwell and Laughlin (2001) predicted that a mean firing rate of 4Hz results in the consumption of 3.29 x 10\textsuperscript{9} ATP/neuron/sec. Only 13% of the energy was required for the resting potential, which accounted for the state of the membrane 99.6% of the time. APs propagating on the membrane accounted for 47% of the ATP consumed the other 0.4% of the time. Postsynaptic receptors were calculated to consume 34% whereas glutamate
recycling and presynaptic calcium entry each consumed 3%. Indeed this modeling of energy consumption based on anatomical and physical data supports the idea that a comparatively small amount of energy is required to maintain the vegetative metabolism of a neuron. It also highlights the validity of the postulate that down-regulation of signaling is an adaptive response to conserve energy in times of limiting oxygen.

Action potentials, such as those seen in axons, are required to send information over long distances. Action potentials are required to threshold out the noise, which would otherwise accumulate in a graded potential over long distances by the random opening of voltage-gated channels (Laughlin 2001). Axons threshold out noise by generating signals that are much larger and far sharper than the noise inherent in the axon, allowing signals to maintain their integrity over long distances. Although expensive, action potentials better ensure that information will not be lost or degraded during its propagation down the length of the axonal transmission line. Action potentials require that an electrochemical gradient be established across the membrane of a neuron. The Na\(^+\)/K\(^+\) ATPase contributes to maintaining the Na\(^+\)/K\(^+\) gradient across the membrane by pumping three Na\(^+\) ions out for every two K\(^+\) ions into a neuron at the expense of one ATP per cycle whereas the potassium leak channels are constitutively open allowing potassium to diffuse down its concentration gradient, thus setting the resting membrane potential. During the action potential ions move towards their electrochemical equilibrium and afterwards must be actively pumped against their concentration gradients in order to maintain the ability of the membrane to propagate action potentials.
Any estimation of spike train cost requires an estimate of the cost of its components, namely the action potentials. Previous estimates of AP cost based on the giant squid axon assumed a four-fold increase in Na\(^+\) current was necessary to depolarize the plasma membrane compared to the depolarization of a pure capacitor (Alle et al. 2009). Typically an estimate for the metabolic cost of an action potential is made by a theoretical calculation of the number of Na\(^+\) ions required to produce the same membrane depolarization as an action potential recorded experimentally. The number of Na\(^+\) ions calculated can then be used to estimate the number of pump cycles the sodium potassium ATPase will require to reestablish the resting potential (Moujahid et al. 2011). Since the Na\(^+\)/K\(^+\) ATPase requires one ATP per cycle, an estimate of the number of cycles is also an estimate of the metabolic energy required to redistribute the ions that moved during the action potential against their thermodynamic equilibrium. This type of estimate however is flawed as it can either grossly underestimate or grossly overestimate the cost of action potentials. By dividing ion fluxes into three separate components during an action potential, Crotty and Levy (2007) demonstrated that a large “overlap” of Na\(^+\) and K\(^+\) currents exists during an action potential, referring to the current as “neutralized” since it had no electrical effect. They also reported that the neutralized component was generally more costly than those exhibiting an electrical effect.

Recent studies utilizing model neurons have considered the relative contribution of current overlap to the metabolic energy cost of APs. These studies have shown that the most energy-efficient APs have a minimal overlap whereas energy-inefficient neurons have substantial overlaps. New estimates based on current overlap suggest the efficiency
of APs in hippocampal mossy fibers is higher than previously thought. The new estimate of AP efficiency in mossy fibers is owing to a fast Na\(^+\) current decay coupled with a delayed K\(^+\) current onset. Alle et al. (2009) found that APs in mossy fiber axons are only 1.3 times the theoretical minimum required to depolarize a pure capacitor, compared with a previous estimate of 4 times the theoretical minimum based on giant squid axon biophysics. Using model neurons, Sengupta et al. (2010) showed that the metabolic cost of an action potential varies up to 22-fold through differences in overlap and height of action potentials. They also showed that energy consumption was increased 10-fold without changing the shape of the action potential recorded simply by altering the overlap between sodium and potassium currents.

Other properties of the action potential that increase their metabolic cost include conduction velocity and frequency. Sangrey and Levy (2005) assessed the cost associated with the timely arrival of action potentials at their point of destination and found that any benefits associated with increased velocity are mitigated by a substantial metabolic penalty. They showed that the metabolic demand which velocity placed on the Na\(^+\)/K\(^+\) pump more than doubled throughout the biological range of velocities. Owing to the inherent cost of conduction velocity it has been suggested that large axon diameter evolved not to support high velocities but to minimize the cost of propagating action potentials at high velocity (Crotty et al. 2006). It has been shown that the rate of ATP consumption associated with the pumping out intracellular Na\(^+\) ions grows non-linearly with frequency (Karbowski 2009). Thus, a reduction in the number of high frequency
APs propagated along the length of an axon may result in a savings that grows non-linearly with a linear decrease in AP frequency.

Cost/benefit analysis of spike trains in high performance cells

A true cost/benefit analysis of spike trains is an evaluation of the cost of information. One needs to know both the cost of transmitting a spike train and the amount of information contained within a spike train (Moujahid 2011). Niven et al. (2007) found that membrane conductance increases supralinearly with maximum information rate such that information is more expensive in higher capacity cells. They suggested that the fixed cost of maintaining a cell ready to signal increases with its maximum rate. Maintaining the ability to transmit signals quickly with high temporal fidelity requires that a membrane has an increased number of conductances and is therefore leakier by nature. Increased leakiness places higher demands on the Na⁺/K⁺ pump and the ATP supply needed to run the pump. Therefore maintaining high performance during times of energetic stress may put a cell at risk of damage. It has been suggested that neurons obey the law of diminishing returns, where excess functional capacity is severely penalized by increases in energetic costs (Niven and Laughlin 2008). The enlargement of sensory organs and the afferent fibers involved in demanding behavioural tasks of particular importance and the converse reduction of redundant structures, suggest that large structures perform better but cost more (Niven et al. 2007).
While there is a long established view that increased axon diameter has evolved to support conduction velocities and a more recent view that increased axon diameter has evolved to reduce the cost of conduction velocity, there is yet another view suggesting that increased axon diameter has evolved to support a larger number of terminal arbors and the larger number of active zones necessary to transfer information at higher rates (Perge et al. 2009). It has been shown that large diameter axons are more vulnerable to hypoxic insults than smaller diameter axons (Peasley and Shi 2002). It has been suggested that this phenomenon is owing to the fact that larger diameter axons are more energy-demanding than smaller diameter axons and that large diameter neurons may be sensitive to the return of oxygen as they regain some function upon reoxygenation (Pryor and Shi 2006).

*Spike train propagation in a high performance visual interneuron*

The locust, *Locusta migratoria*, possesses a well characterized and compact neural network highly specialized for transformation of sensory signals into motor commands critical to predator avoidance behaviours. The descending contralateral movement detector (DCMD) is a visual interneuron within this network and it is responsible for the faithful and timely transmission of a spike train generated in the brain in response to an object on a collision course. The DCMD has a large diameter axon (~15-17 µm) and makes a myriad of synaptic connections in both mesothoracic and metathoracic ganglia (Fotowat and Gabbiani 2011). In the mesothoracic ganglion axonal
arbor of the DCMD have been shown to make synaptic connections with a pair of motor neurons (motor neurons no. 84) involved in evasive flight movements (Simmons 1980), a pair of auditory G-interneurons (Pearson et al. 1980) and a pair of large interneurons (C-neurons). The firing of C-interneurons results in the co-contraction of hindleg flexor and extensor muscles achieved by the co-activation of innervating motor neurons (Pearson and Robertson 1981), implicating DCMD’s involvement in the execution of escape jumps. Axonal arbors from each DCMD form synaptic connections with a pair of multimodal inhibitor interneurons (M neurons) located in the metathoracic ganglion. M neurons receive excitatory input from the DCMD as well as auditory, tactile and proprioceptive excitatory inputs, which influence the generation of an inhibitory signal destined for flexor motor neurons in the mesothoracic and metathoracic connectives (Pearson et al. 1980). Direct connections between axonal arbors from each DCMD and a pair of fast extensor tibiae (FETi) motor neurons have been demonstrated and DCMD activity has been shown (directly or indirectly) to elicit an electrophysiological response in several other motor neurons including: the anterior coxal adductor motor neuron (AAdC), anterior inhibitor (AI), common inhibitor (CI), and posterior inhibitor (PI) (O’Shea et al. 1973). The number of axonal arbors along with DCMD’s speed of conduction (3.1 m/s) and the high frequency spikes that it supports (>200 Hz) (Burrows and Rowell 1973) fit the predictions of Perge et al. (2009) that axons of large diameter are designed to transfer information post-synaptically at high rates.

The spike train transmitted by the DCMD is generated in the lobula giant movement detector (LGMD), a visual interneuron located in the third neuropil of the
optic lobe (Rind 1983). The LGMD receives ~15000 elementary visual inputs, responding selectively to objects approaching on a collision course (Jones and Gabbiani 2010). In response to the inputs generated by objects on a collision course, or their two-dimensional simulations (looming stimuli), the LGMD generates a train of action potentials in which the firing rate gradually increases, peaks and decreases prior to collision. The LGMD projects its axon from the optic lobe into the ipsilateral protocerebrum where it makes a robust chemical and electrical synapse with the DCMD, a connection which transmits action potentials in a 1:1 correspondence (Rind 1983). The diameter of the DCMD soma (45-50 µm) is thought to be one of the largest in the brain and sends a large diameter axon dorsomedially across four ganglia (suboesophageal, prothoracic, mesothoracic and metathoracic) and 4 lengths of connective (O’Shea et al. 1973). Recent experiments with freely moving locusts have shown the DCMD to be important for the transmission of information, coding the “precise” firing time of FETi motor neurons. (Fotowat et al. 2011). Maintaining the membrane potential near threshold values would facilitate the transmission of information quickly, efficiently and accurately.

Therefore the cost associated not only with transmitting a dense amount of information, but with maintaining the membrane resting potential within an optimal range before during and after spike train transmission merits investigation.
**Hypoxia-Tolerance in the locust**

The hypothesis that the possession of a high metabolic rate precludes any type of tolerance towards hypoxia holds true for both mammals and insects when considering abrupt onsets of anoxia. However, mammals and insects which possess high metabolic rates do in fact differ both in terms of their ability to recover from anoxia and their ability to cope with graded hypoxia (Wegener and Moratzky 1995). Insects of the order Orthoptera are able to recover from hours, days and even months without oxygen (Hoback and Stanley 2001). The locust *Locusta migratoria* is tolerant of oxygen deprivation, withstanding hours of water submersion and is able to survive 4 hours in an atmosphere of pure nitrogen (Armstrong *et al.* 2009; Wegener and Moratzky 1995). The mechanisms behind the inherent hypoxia tolerance of insects are little understood and of potential relevance to the biomedical field (Harrison *et al.* 2006).

Although few species have been studied in detail, survival times are linked to ecological situations in which individuals are exposed to anoxia/hypoxia for example during periods of flooding or life/flight at high altitudes (Hoback and Stanley 2001). The ability of insects to sustain a stable, albeit reduced, metabolic rate during hypoxia is thought to involve a suppression of metabolically demanding processes along with a concomitant switch from aerobic metabolism to anaerobic metabolism (Hochachka *et al.* 1993; Wegener 1993). A reduced metabolic demand must be accompanied by behaviours that are less metabolically demanding, however this is not always an option for the
animal. Using tethered flying locusts Rascon and Harrison (2005) demonstrated that hypoxia tolerance in the resting locust may be due to the excess oxygen delivery capacity reserved for bouts of flying. Flight, especially at high altitudes may broach the safety margins of oxygen delivery threatening the animal with hypoxic insults to its tissues.

*The AMP-activated protein kinase cascade; balancing supply and demand*

The membrane potential functions as a battery whose charge is maintained by electrogenic pumps. Similarly the high ratio of ATP:ADP can be thought of as a battery in its fully charged state maintained by the oxidation of reduced organic hydrocarbons. Catabolism charges the battery, while anabolic pathways and most other tasks performed by the cell discharge the battery. In spite of the fluctuating conditions experienced by the average cell it maintains the high ratio of ATP:ADP within rather narrow limits. A decrease in the ratio of ATP:ADP is either caused by a reduction in ATP production owing to the reduced oxidation of fuels (hypoxia) or increased ATP consumption (sensory signaling). In order to maintain the battery that drives cellular processes in its fully charged state it is essential that ATP consuming and producing processes be maintained in balance at all times.

AMP-activated protein kinase (AMPK) plays a central role in balancing the activities of ATP consuming and producing pathways. The AMP-activated protein kinase is “ultrasensitive”, i.e. kinase activity (output) of this molecule is three orders of magnitude greater than its input, a small change in the concentration of AMP leads to a
large change in AMPK activity (Hardie et al. 2006; Hardie 2011). AMPK is also localized close to the plasma membrane and its activation is known to reduce both large conductance and background conductance inducing membrane depolarization (Kumar 2007). The location of the AMPK as well as its sensitivity to minor fluctuations in the ATP:AMP ratio make it an attractive candidate for investigations into the down-regulation of membrane permeability and function in response to hypoxia. AMPK activity has been shown to be necessary and sufficient for changes seen in motor pattern generation in response to metabolic stress (Rodgers-Garlick et al. 2011). It is therefore of interest to utilize other neuronal models for the determination of the membrane conductances behind performance changes observed during AMPK activation.

*The role of hyperpolarization-activated cyclic nucleotide-gated channels in neuronal performance under ideal and hypoxic conditions*

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are non-selective cation channels (Jegla et al. 2009) that generate a current referred to as queer ($I_q$), funny ($I_f$) or hyperpolarizing ($I_h$) because HCN channels, unlike other potassium channels, are activated by hyperpolarizing rather than depolarizing currents and they generate a depolarizing rather than a hyperpolarizing current. HCN channels conduct both Na$^+$ and K$^+$ with permeability ratios of ~1:4 respectively. Another peculiarity of the HCN channels is their dual dependence upon voltage and cAMP (DiFrancesco 1999).
HCN channels are thought to contribute to the resting membrane potential (Doan and Kunze 1999; Baginskas et al. 2009) and the activation of HCN by hyperpolarizing current generates an antagonistic depolarizing current driving the membrane potential back towards the action potential firing threshold (Dyhrfjeld-Johnson et al. 2009). Activation of HCN is facilitated directly by cAMP in a phosphorylation-independent manner whereby the binding of cAMP to the HCN channel’s cyclic nucleotide binding domain (CNBD) shifts the voltage dependence of channel activation toward more positive membrane potentials (Biel 2009). The binding of cAMP to the HCN channel also affects the kinetic properties of the channel, speeding up activation and slowing down deactivation (Wicks et al. 2011).

In neurons highly specialized for the precise analysis of temporal acoustic information, \( I_h \) has been shown to improve the temporal precision of input processing by lowering input resistance, shortening time constants and reducing temporal summation (Koch and Grothe 2003). \( I_h \) has also been shown to be important in the propagation of bursts of APs having spike intervals of ~2ms. Selectively blocking HCN reduces conduction velocity and causes propagation failures at branch points (Baginskas et al. 2009). It has been suggested that activity-induced hyperpolarization of the axonal membrane caused by the Na\(^+\)/K\(^+\) pump is counteracted by the depolarizing current of \( I_h \), which secures conduction reliability by the reduction of activation threshold (Soleng et al. 2003).

Neuronal performance can be modulated by biogenic amines during times of social stress (dominant/subordinate relationships) or during behaviours requiring
enhanced performance such as flight. Although counterintuitive in terms of generating an adaptive behaviour, dominant crayfish have a more excitable lateral giant escape reflex than subordinates. Krasne et al. (1997) suggest that unlike for dominant animals it is more adaptive for subordinate animals to engage motor circuitry involved in long latency non-reflex or “voluntary” responses rather than short latency giant fiber-mediated “reflex” responses. In subordinates serotonin inhibits the lateral giant reflex whereas it increases this reflex in dominant animals. In the case of social encounters the adaptive response of the subordinate is to produce an unpredictable non-stereotyped behaviour however, in the case of flight, fast reflexes are of extreme detriment. Weisel-Eichler et al. (1996) showed that octopamine, known to increase excitability of insect giant interneurons, when bath applied to major synaptic sites involved in flight circuitry lowered the threshold of wind-evoked initiation of flight.

The monoamines dopamine, octopamine and serotonin have been shown to modulate neuronal \( I_h \) by shifting its activation potential to a more depolarized level, thus shaping motor pattern output of a discrete neural circuit (Peck et al. 2006). Additionally, Ballo et al. (2010) present evidence of the neuromodulator dopamine affecting the activation properties of an axonal HCN channel through a cAMP-dependent phosphorylation-independent manner. Therefore it seems that modulation of the intrinsic excitability of neurons through aminergic modulation of \( I_h \) may have a central role in the generation of adaptive behaviour.

It has been suggested that the intrinsic excitability of neurons may in fact be a contributing factor to the observed differences in vulnerability observed among striatal
neurons in response to cerebral ischemia (Deng et al. 2008). Gao et al. (2006) demonstrated that in hypoxia-sensitive neurons, $I_h$ was completely abolished in less than 3 minutes of hypoxia owing to a negative shift in HCN’s voltage dependence of activation and that this effect of hypoxia could be reversed within 5 minutes of reperfusion with normoxic saline. Deng et al. (2008) showed that it was possible to up-regulate $I_h$ amplitude using the cAMP analogue (Br-cAMP) in both hypoxic and control conditions however, using the cAMP pathway inhibitor (RP-cAMP) it was only possible to down-regulate $I_h$ under normoxic conditions. These results advocate for the argument that under hypoxic conditions the reduced permeability of neurons is a major defense strategy. HCN’s neuroprotective role has also been demonstrated by the application of the $I_h$ blocker ZD7288 to hippocampal slice cultures preventing cell death when applied either during or after oxygen and glucose deprivation insults (Ray et al. 2003).

**Hypothesis**

I am interested in how animals are able to balance the supply and demand of energy. Balancing supply and demand has not only to do with replenishing spent fuels, it also has to do with the ability to burn fuels quickly and efficiently, at rates that can support the energy demand required for the adaptive behaviour of an organism as a whole. A certain amount of constant energy is needed to maintain the order of complex structures. I am interested in the strategies cells employ to maintain the structural and thus functional integrity of their highly-organized and tightly-regulated components, in
times of reduced energy production. Neurons are especially vulnerable to reductions in energy supply as they are in constant need of energy to maintain ionic gradients across their plasma membranes. The dissolution of these gradients can spell disaster for a neuron or indeed any cell as it triggers a number of negative reactions within the cell that destroy the complex structure of the cells often leading to cell death.

It thus seems perfectly logical to conceive that reducing permeability of neurons during energetic stress conserves energy and prolongs the dissolution of the ionic gradients generated for electrochemical communication between neurons. Reducing the permeability required for electrochemical signaling subsequently decreases the ability of the neuron to communicate. Using the DCMD as a model of a high performance neuron, having a highly permeable membrane to support the high frequency action potentials and preserve the temporal nature of APs across long distances, I hypothesize that the adaptive measure taken by the DCMD during times of reduced energy supply is to reduce its permeability and thus its signaling capabilities. I assessed the performance of the DCMD during periods of minimal energetic stress and periods of significant energetic stress. I also assessed the ability of octopamine to affect performance during periods of significant energetic stress. I investigated the possibility of manipulating axonal performance by activating pathways involved in the balancing of ATP consuming and producing processes, namely the AMPK pathway. In an attempt to identify conductances necessary for high performance in DCMD axon I selectively blocked the HCN channel during minimal energetic stress.
CHAPTER 2: MATERIALS AND METHODS

Animals and Preparations

Adult male locusts (*Locusta migratoria*) 3-5 weeks past the imaginal ecdysis were obtained from a crowded colony located within the Department of Biology, Queen’s University. Colony room and cage lights were set to a 12:12 light:dark cycle resulting in a mean daily temperature of 26.6 °C ±1.2 °C. When removed from the colony animals were placed into a 1L ventilated container until the time of dissection.

For all experiments animals had their thoracic appendages (legs and wings) as well as the dorsal surface of the pronotum removed. A dorsal midline incision was then made allowing the animal’s thoracic cavity to be pinned open by a gentle spreading of the thoracic cuticle. The gut and other obstructing tissues and cuticles were then removed to expose the ventral nerve cord within the thorax (semi-intact preparation) (Figure 1i).

Normoxic preparations were perfused with standard locust saline [147 mM NaCl, 10 mM KCl, 4 mM CaCl₂, 3 mM NaOH, 10 mM HEPES (pH = 7.2) ] containing an additional 1mM of glucose. Saline was delivered to normoxic preparation by way of a gravity feed, which flowed at a rate of 6 mL/min. A glass inlet pipette delivered saline into the preparation just posterior to the head of the animal on the side opposite to
Figure 1. Experimental overview. i) Animals were dissected dorsally to expose the ventral nerve cord. Combinations of two or three suction electrodes were placed at positions a-b, b-c or a-b-c along the DCMD (labeled green). ii) A simulated looming stimulus was represented as an expanding black disc projected on a translucent screen 7cm from the animals left eye. iii) The LGMD responds preferentially to the looming stimulus by generating a train of action potentials transmitted to the DCMD in a 1:1 correspondence. iv) The spike train generated over a 3 second period during the appearance and expansion of the looming stimulus was recorded from the DCMD at a minimum of two and maximum of three positions along its length. v) Suction electrode recordings produced triphasic waveforms for individual action potentials.
stimulus presentation. Saline was allowed to exit from the animal by flowing over both the lateral remnants of the pronotum allowing for a dry environment around abdominal spiracle openings. For the same reason normoxic preparations were pinned to a small island of elevated corkboard to insuring that any overflow of saline from the abdomen would not block airflow into the spiracles. Normoxic conditions as inferred from DCMD performance were maintained over long periods of time when the tracheal tubes running parallel to the ventral nerve cord and their branches ventral to and supplying the ganglia were left intact (Figure 2i).

All drugs ((±)-octopamine hydrochloride, metformin hydrochloride and ZD7288 hydrate) were obtained from Sigma-Aldrich Company (Sigma-Aldrich Canada Ltd. Oakville, Ontario). Salines containing octopamine (OA) (0.1mM), metformin (100 mM) and ZD7288 (0.1mM) were freshly made every day. Glucose (0.1mM) was added to all salines used in normoxia experiments and was absent in all hypoxia experiments.

Hypoxic preparations were perfused with standard locust saline delivered to the preparation with a 2 mL pasteur pipette. Saline level in the animal was maintained at a level comparable to the normoxic preparations by actively replenishing saline displaced by abdominal pumping of the animal. No care was taken to prevent blockage of abdominal spiracle openings by the overflow of saline. As a means of stabilizing electrophysiological recordings a metal plate was gently placed underneath the mesothoracic and metathoracic ganglia. A secondary consequence of using the metal was
Figure 2. Normoxic and hypoxic nerve cord preparations. i) Normoxic conditions in the ventral nerve cord were achieved in dissections that left the tracheal system intact. ii) Hypoxic conditions in the ventral nerve cord were created when a metal plate was placed gently beneath the mesothoracic and metathoracic ganglia damaging the tracheal tubes supplying the ganglia.
the creation of hypoxic nerve cords through a severing of the tracheal oxygen supply of both ganglia (Figure 2ii). While oxygen levels surrounding the DCMD were not measured directly, all tracheal connections to the ventral nerve cord were eliminated caudal of the prothoracic ganglion as confirmed by anatomical investigation.

Evoked-stimulus and Measured Response

The simulated looming stimulus was created in Adobe Flash and consisted of a series of 300 images of black disks having progressively larger diameters, one every 10 ms over a 3 s period. The image was projected on a translucent screen 90 degrees perpendicular to and 7cm away from the animals left eye (Figure 1ii). The object simulated was a black disc with a diameter of 3.75 cm moving directly towards the locust’s eye at a speed of 1m/s (Figure 3i). At 500 ms to a simulated collision the diameter of the disc projected on the screen had the same angle of subtense as an object with the diameter of 3.75 cm has 50 cm from the locust’s eye at 500 ms to actual collision (Figure 3i, ii). The image was projected using a DV11 Optima digital projector with a horizontal refresh rate of 100 kHz and vertical refresh rate of 120 Hz.

The LGMD ipsilateral to the stimulated eye (Figure 1iii) responds preferentially to looming stimuli. The firing rate within the spike train gradually increases, peaks and then quickly decreases in response to looming stimuli (Figure 1iv). The LGMD then
Figure 3. Two-dimensional representation of the looming stimulus. The size of the disc projected on the screen at any given point in time is represented by the angle of subtense its edge made with the animals eye.
transmits the spike train to the DCMD in a 1:1 correspondence. The spike train can be detected by extracellular recordings from any point along the length of DCMD contained within the connectives of the nerve cord (Figure 4). Extracellular recordings were made at three separate points along the ventral nerve cord contralateral to the stimulated eye using glass suction electrodes. Two recordings were made in the length of axon contained within the prothoracic-metathoracic ganglion and one was made in the mesothoracic-metathoracic connective (Figure 1v and Figure 2i, ii).

Experimental Procedure

With suction electrodes placed at positions (a and b), (b and c) or (a, b and c) (Figure 1i and Figure 2i, ii) looming stimuli were presented at 1, 5, 10, 20, 25, 35, 45, 55 and 65 minutes (Figure 5i, ii). Hypoxic control and hypoxic octopamine-treated groups both received 5 pasteur pipettes or 10 mL of saline at 15 minutes. From 15 minutes until the end of the experiment the octopamine-treated group received the standard saline containing 0.1 mM octopamine (Figure 5i). The normoxic control group received a steady flow rate (6 mL/min) of standard locust saline containing 1 mM glucose throughout the experiment. For metformin and ZD7288 treated groups (Figure 5ii) the flow through of 6 mL/min was stopped after the recording made at 25 minutes at which point standard saline containing 1 mM glucose and either 10 mM metformin or 100 μM
Figure 4. Visualization of the scale of the DCMD axon relative to the connective. The DCMD axon labeled green is ~17µm in diameter and is located dorsomedially in the connective immediately beneath the connective sheath. Obtaining an extra-cellular recording from DCMD axon is highly repeatable and recordings are stable over long periods of time.
Figure 5. Experimental/pharmacological time course. i) Octopamine experiments; baseline recordings of looming stimuli generated spike trains were recorded at 1, 5 and 10 minutes. Saline was changed to a saline containing 0.1 mM Octopamine at 15 minutes and subsequent recordings were taken at 20, 25, 35, 45, 55 and 65 minutes. ii) Metformin and ZD7288 experiments; baseline recordings were taken at 1, 5, 10, 20 and 25 minutes. Saline containing 10 mM Metformin or 100 μM ZD7288 was applied between 26 and 46 minutes and recordings during treatment were taken at 35 and 45 minutes. The preparation then received a continuous washout from 46 minutes until the completion of the experiment. Recordings during the washout period were taken at 55 and 65 minutes.
ZD7288 was pipetted into the preparation. The saline level was actively monitored and maintained. Following the recording at 45 minutes the saline flow through was recommenced at a rate of 35 mL/min for 5 minutes to ensure an adequate wash out of drugs. At 50 minutes the flow rate was reduced to 6 mL/min allowing a 5-minute period with a reduced flow rate before subsequent recordings.

All data were acquired using Molecular Devices software Clampex 10.2 and stored digitally on computer. Analog recordings from suction electrodes a, b and c were amplified using A-M systems differential AC amplifier (model 1700). Electrodes (a, b and c) were inputs for the amplifier channels (1, 2 and 3) respectively. For each channel low frequency cutoffs were set to 0.1 Hz and high frequency cutoffs were set to 10 kHz. The analogue output signals from the three separate amplifier channels constituted three separate inputs into the digitizer (Digidata 1440 A). A fourth input channel into the digitizer was an audio signal designed to signal the appearance of the first and last frames at 0 ms and 2930 ms of the simulated looming stimulus. This allowed for the identification of spikes generated during the presentation of the looming stimulus. Digitized signals were sampled by clampex 10.2 software continuously in the gap-free event mode at a rate of 250 kHz equivalent to 1 sample every 4 μs.
**Data Analysis**

Files containing multiple spike train recordings were fragmented into files containing single spike trains generated at 1, 5, 10, 20, 25, 35, 45, 55, and 65 minutes with the use of Clampfit 10.2 software. Using Dataview 7.5.3 software, extracellular records of action potentials were initially identified by placing a horizontal cursor at a voltage near the peak negative voltage of the second phase of the triphasic waveform such that all DCMD APs within a spike train could be simultaneously identified. Figure 6i indicates an approximation of the point in the triphasic waveform at which APs were identified. In dataview the relative timing of neighbouring AP events recorded from the same electrode (frequency/inter-spike-interval) and the relative timing of the same AP event recorded in different electrodes (conduction delay) were measured. Instantaneous frequency of an AP event was defined by the duration of time between the event and the AP that preceded it. Inter-spike-interval was defined by the time span between neighbouring events.

Individual responses to looming stimuli were generated by plotting the time delay between the recording of an AP event in the anterior electrode and the posterior electrode (a to b) (b to c) or (a to c) against the instantaneous frequency of the same AP event measured in the posterior electrode. Individual responses were plotted using Sigma Plot 11.0.
Figure 6. Information available from triphasic action potentials. a) Analyzing one point in time from the same stage of each action potential allowed for the determination of instantaneous frequencies and interspike intervals of APs from one source electrode as well as conduction delay and propagation fidelity using two electrodes. b) Determining the time and voltage coordinates for the peak positive voltage of the 1st (yellow) and 3rd (blue) phases and the peak negative voltage of the 2nd phase (green) allowed comparisons of the triphasic wave forms of action potentials generated before during and after pharmacological manipulations. Comparisons provided insight into differences in monophasic waveform.
Mean relative conduction delays (MRCD) and standard error for frequency bins of <100 Hz, 100-200 Hz and >200 Hz were calculated for the normoxic control, hypoxic controls and OA-treated hypoxic groups. Responses to looming stimuli considered were at the time points 10, 25, 45 and 65 minutes otherwise referred to as 5 minutes before OA application, 10, 30 and 50 minutes of OA application. MRCD and standard error were also calculated using the same frequency bins for normoxic metformin and normoxic ZD7288 treated groups. Time points considered were 25, 45, and 55 minutes otherwise referred to as before during and after treatment. MRCD was similarly determined for the normoxic control group at 55 minutes. The delays and frequencies were those previously determined for individual responses. Delays were made relative for analyses of AP propagation in hypoxic nerve cords by dividing all AP delays at 10, 25, 45, and 65 minutes by the shortest delay at 10 minutes. Delays were made relative for analyses of AP propagation in normoxic nerve cords by dividing all AP delays at 25, 45, and 55 minutes by the shortest delay at 25 minutes. MRCD and standard error were calculated using Sigma Plot 11.0. The delay used for AP propagation comparisons of treated and untreated normoxic nerve cords was the delay measured for the length of axon within the ganglion (b-c), owing to the lack of control normoxic recordings from the length of axon contained within the connective (a-c). Delays considered for hypoxic groups (treated and untreated) were from electrodes a to b and b to c.

Spike train fidelity was assessed by dividing the spike count of the posterior electrode by that of the anterior electrode for recordings made from the length of DCMD
axon contained within the prothoracic-mesothoracic connective (electrodes a and b) and those made from the length of axon spanning the mesothoracic ganglion (electrodes b and c). Maximum relative conduction delay was defined as the greatest time delay observed between anterior and posterior electrodes for a single AP event within a spike train. Means and corresponding standard errors were calculated for the percent fidelity of action potentials within a spike train and the maximum relative conduction delay of a single AP event within a spike train using Sigma Plot 11.0.

Mean spike count, mean peak frequency and their corresponding standard errors were calculated for normoxic control, hypoxic control, OA-treated hypoxic, normoxic metformin treated and normoxic ZD7288 treated groups. Spike count was defined as the number of spikes occurring during the coding window of the 3 second looming stimulus and peak frequency was the highest instantaneous frequency recorded within a spike train. The time points considered for respective treatment groups were the same as those considered in mean relative conduction delay calculations. Electrode b was used for mean spike count, mean peak frequency and standard error calculations in order to achieve the highest sample size possible. Means and standard errors were calculated using the quick transform function in Sigma Plot 11.0.

Histograms generated to display changes in inter-spike-interval for normoxic groups (treated and untreated) before, during and after treatment were constructed by grouping events into integer bins of 1ms. Events with ISIs that fell between 0 to 1 ms
were considered as 0 ms and 1 to 2 ms were considered as 1ms etc. from 0 to 10 ms. Histograms were generated using the quick transform function in Sigma Plot 11.0.

Estimated delay of APs between the LGMD (source) and the FETi (destination) were computed for normoxic preparations treated with either metformin or ZD7288. The nerve cord was dissected out of the animal from the posterior end of the metathoracic ganglion up to and including the brain and the optic lobe. The locust’s central nervous system was then splayed out on 1mm Cartesian graphing paper and the distance between (LGMD and FETi), electrodes (a and b), (b and c) and thus (a and c) were measured. The shortest delay between electrodes at 25 minutes for each animal (i.e. the same delay used to make delays relative in the analysis of MRCD) was converted from a delay between the spacing of electrodes to an estimated delay between the LGMD and FETi. The mean delay between LGMD and FETi was then calculated separately for metformin and ZD7288 treatment groups. The mean delay between LGMD and FETi was calculated once for metformin and once for ZD7288 groups. The mean delay calculated for a specific treatment group was then multiplied by individual relative conduction delays before during and after treatment for frequency bins of < 100 Hz, 100-200 Hz and > 200 Hz. This series of calculations generated sets of data for estimating the time delay between LGMD and FETi for low, medium and high frequency bins before, during and after treatment. Means and standard errors for the estimated time delays were determined using Sigma Plot 11.0.
Events of similar frequencies (lowest frequency and highest frequency) within a spike train were also compared for their differences in triphasic waveform Figure 6. (ii). Three time/voltage coordinates were determined for triphasic waveforms using clampfit 10.2 software. Placement of three vertical cursors (1 and 3) at the peak positive voltage of the first and third phases and (2) at the peak negative voltage of the second phase allowed for comparisons of relative changes in duration and amplitude between the three coordinates identified as well as the amplitude changes of the first and third coordinates relative to the baseline of 0 mV. Temporal measures of comparison included; AP duration (xa), duration of the last half of the first phase and first half of the second phase (xb) and duration of the last half of the second phase and the first half of the third phase (xc). Amplitude measures of comparison included the amplitude of the first phase (ya), the amplitude of the positive peak of the first phase to the amplitude of the negative peak of the second phase (yb), the amplitude of the negative peak of the second phase to the positive peak of the third phase (yc) and the amplitude of the of the third phase (yd) (Figure 6ii). Means and standard errors for all measures of comparison were calculated to determine specific aspects of the triphasic AP affected by pharmacological treatments with either metformin or ZD7288.

Two dimensional raster plots were generated using the event-triggered scope view application in DataView. Two dimensional raster plots were generated for electrode b before during and after treatment with either metformin or ZD7288. The voltage range for the colour spectrum of the 2D raster plots were set to integers encompassing the voltage range of all three spike trains recorded before, during and after a given treatment.
Statistical analyses performed were all two-way repeated measures Analysis of Variance (ANOVA) with student t-tests, performed using SigmaPlot 11.0 software.

Significance was assessed at $p < 0.05$. 
CHAPTER 3: RESULTS

*Spike train propagation is compromised in hypoxic nerve cords*

Normoxic nerve cords were considered to be those where the ventral longitudinal tracheal trunks running parallel and lateral to the ventral nerve cord were intact and inflated (Figure 2i). Ventral to the thoracic ganglia, lateral tracheal trunks connecting longitudinal trunks send two short tubes directly dorsal connecting the lateral trunks with thoracic ganglia. Care was taken when removing tissue overlying the nerve cord not to sever the hidden connection between ganglia and the lateral tracheal trunks. Hypoxic nerve cords were considered to be those where a metal plate was placed ventral to the mesothoracic and metathoracic connectives severing their connections to the longitudinal trunk trachea (Figure 2ii).

In normoxic nerve cords, conduction delays for any given frequency were stable over the recoding period of 65 minutes (Figure 7v). Hypoxic nerve cords demonstrated an additive increase in their conduction delay for spike trains over the duration of recording period (Figure 7i, iii). Mean relative conduction delays (MRCD) were calculated for frequency bins of < 100 Hz (low) 100-200 Hz (medium) and > 200Hz (high) frequency action potentials. Calculations were performed for the time points 10, 25, 45 and 65 minutes. Normoxic controls for the hypoxic nerve cord were only performed for the length of axon within the meothoracic ganglion (delay between
Figure 7. Individual responses to loom recorded from normoxic, hypoxic and hypoxic OA treated nerve cords. Instantaneous frequency of APs plotted against their respective conduction delay for hypoxic control (i, iii), hypoxic octopamine treated (ii, iv) and normoxic preparations (v), sampled over the time course of 65 minutes. Electrodes a and b were placed on the prothoracic-meso thoracic connective and electrode c was placed on the mesothoracic-metathoracic connective
electrodes b and c). Therefore, comparisons of normoxic and hypoxic nerve cords are here only considered for the length of DCMD axon spanning the mesothoracic ganglion. High frequency bins of APs had a significantly longer MRCD compared to both low and medium frequency bins at all time points analyzed. The only difference in MRCD for the normoxic control over the course of the experiment was a difference between high frequency bins at 10 and 65 minutes (p < 0.001) (Figure 8i, iv).

At 10 minutes of recording and for all subsequent recordings (Figure 8i, ii, iii, iv), there was a significant difference between high frequency bins of MRCD in normoxic and hypoxic nerve cords (p < 0.001). Unlike the normoxic nerve cord, in the hypoxic nerve cord at 10 minutes there was a significant difference between medium and low frequency bins (p < 0.005). Five minutes before treatment there was no difference between OA-treated and untreated preparations, at which point both preparations displayed a similar difference between medium and low frequency bins (p < 0.001) for delays recorded from axon contained within the mesothoracic ganglion. At 25 minutes into recordings and for all subsequent recordings (45 and 65 minutes) (Figure 8ii, iii and iv) there was a significant difference between medium frequency bins of MRCD of normoxic and hypoxic nerve cords (p < 0.001). By 25 minutes and throughout all subsequent recordings there was a significant difference between medium and low frequency bins of MRCD in hypoxic nerve cords (p < 0.001). In the hypoxic nerve cord there were no significant differences when comparing low, medium or high frequency bins at 45 minutes with low, medium or high frequency bins at 65 minutes (Figure 8iii, iv).
Figure 8. Octopamine attenuates the time-dependent hypoxia-induced rundown of DCMD axonal conduction velocity for the length of axon contained within the mesothoracic ganglion. Mean relative conduction delay (MRCD) for low (<100Hz) medium (100-200Hz) and high frequencies (>200Hz) were determined for five separate treatments (i) 5 minutes before treatment (ii) 10 minutes of treatment (iii) 30 minutes of treatment and (iv) 50 minutes of treatment. For the length of axon within the mesothoracic ganglion (from electrode b to c) treatments included 1) hypoxic control (normoxic nerve cord) 2) an experimental control (hypoxic nerve cord) and an experimental group (hypoxic nerve cord in a 0.1 mM OA bath). For the length of axon contained within the prothoracic-mesothoracic nerve cord (from electrode a to b) the two
remaining treatments 4) and 5) were the same as their respective treatments 2) and 3) for the length of axon within the mesothoracic ganglion.
In preparations with hypoxic nerve cords it was often observed that spikes of high instantaneous frequency recorded in electrode b were not recorded in electrode c and constituted an AP failure in the length of axon contained within the mesothoracic ganglion. Failure of action potentials within a spike train always occurred later in the spike train near what was considered to be the peak-firing rate. Percent fidelity of spike trains was assessed by dividing the number of spikes in the posterior electrode by that of the anterior electrode. APs recorded in the anterior electrode, which were not observed in the posterior electrode, were always APs having the highest instantaneous frequency in the spike train as measured from the anterior recording. Since the relative delay of failed events could not be determined the relative delay of the event with the highest frequency in the posterior electrode was taken as the maximum relative delay. No failures were observed for preparations with normoxic nerve cords. In contrast, failures began to occur by 25 minutes into the recording period of experiments on preparations with hypoxic nerve cords (Figure 9). Failures of APs in hypoxic nerve cords were also tightly correlated with the observed maximum delay in a spike train.

During the course of spike train propagation down the DCMD axon, spike train properties were compromised more so in hypoxic nerve cords than those in a normoxic state. Spike number was not affected in hypoxic nerve cords (Figure 10i) however, the peak instantaneous frequency was. Comparisons of the peak frequency in spike trains measured from normoxic and hypoxic nerve cords reveal significant differences between the two at each time point measured (Figure 10ii). Peak frequency was stable throughout the recording period in normoxic nerve cords. In hypoxic nerve cords, peak
Figure 9. Spike train fidelity in the DCMD axon was compromised within the mesothoracic ganglion of hypoxic nerve cords. Spike train mean percent fidelity and mean maximum conduction delay were determined for four separate treatments (i) 5 minutes before treatment (ii) 10 minutes of treatment (iii) 30 minutes of treatment and (iv) 50 minutes of treatment.
Figure 10. The effect of bath applied octopamine on the spike count and peak frequency of spike trains recorded from the axon of DCMD in hypoxic nerve cords. Bath application of 0.1 mM octopamine had no effect on spike count (i) or peak frequency (ii). Peak frequency of the hypoxic control at 5 minutes before treatment was significantly different than all other times within the control group however no differences existed between OA treated and control for any of the times analyzed.
frequency decreased between recordings at 10 min and 25 min ($p < 0.001$) and then remained at a stable level throughout the recording period. There were no significant differences between the peak frequency of the control and OA-treated hypoxic nerve cords at 10 min.

An estimate of the DCMD conduction velocity for both normoxic and hypoxic nerve cords was made using the conduction delay of APs having the lowest instantaneous frequency. The normoxic delays were taken from APs 25 min into recordings whereas hypoxic conduction delays were taken from APs 10 minutes into recordings. The maximum velocity estimated for DCMD under normoxic conditions was 5.7 m/s and under hypoxic conditions the maximum was estimated to be 3.1 m/s.

*Effects of hypoxia on spike train propagation are not strictly uniform along the entire length of DCMD axon*

Conduction delay increased progressively over the recording period for the length of axon contained within the prothoracic–mesothoracic connective and the length of axon contained within the mesothoracic ganglion (Figure 7i, iii). Unlike the length of axon contained within the ganglion the increase in conduction delay of APs for the length of axon within the connective did not depend on their instantaneous frequency.

At only 10 minutes into the recording period there was a difference in MRCD between low and medium frequency bins for the length of axon contained within the ganglion (Figure 8i) however, it was not until 65 minutes into the recording period (50
min relative to drug application) (Figure 8iv) that there was any difference in MRCD between medium and low frequency bins for the length of DCMD axon contained within the connective. After ten minutes of recording there were no differences in MRCD between the lengths of axon contained within the connective and those in the ganglion (Figure 8i). By 25 minutes and for all subsequent recordings there was a difference (p < 0.001) in MRCD for the high frequency bins of these two separate lengths of axon (Figure 8ii, iii, iv).

No failures in AP propagation were observed in the connective for any of the time points analyzed. Ten minutes into recording there were no differences in mean percent fidelity or mean maximum relative conduction delay. AP failure had occurred 25 minutes into the recording period in the length of axon within the ganglion (Figure 9ii). By 45 minutes and for the last recording at 65 minutes, there was a significant difference in mean percent fidelity of action potentials for the length of axon in the connective and the length in the ganglion (p < 0.001) and (p = 0.008) (Figure 9iii, iv). By ten minutes and for all subsequent recordings there was a significant difference in mean maximum relative conduction delay between the two different lengths of axon (p < 0.001).

Octopamine attenuates the effects of hypoxia in the ventral nerve cord on spike train propagation

From an initial comparison of individual responses to the looming stimulus octopamine appears to have had no effect on AP propagation in the length of axon
contained within the prothoracic-mesothoracic connective (Figure 7i, ii). This observation held true for nearly all of the subsequent analyses for DCMD axon within the connective. There were no differences in MRCD between OA-treated hypoxic connective and its control for any time point or frequency bin (Figure 8i, ii, iii, iv). Similarly there were no differences between mean percent fidelity of spike trains or mean maximum relative conduction delay for any of the times analyzed (Figure 9i, ii, iii, iv). Octopamine, did however, preserve the similarity between low and medium frequency bins of MRCD which became distinct from one another at 65 minutes in the untreated hypoxic connective (p < 0.001) (Figure 8iv).

An initial comparison of individual responses suggests that octopamine was able to stabilize the functioning of DCMD axon within the hypoxic mesothoracic ganglion (Figure 7iii, iv). At all times analyzed there was no distinction between low and medium frequency bins of MRCD in the length of axon within the normoxic mesothoracic ganglion (Figure 8i-iv). After 50 minutes, OA treatment of the hypoxic ganglion resulted in the restoration of the inherent similarity between low and medium frequency bins of MRCD seen in normoxic conditions (Figure 8iv). MRCD for the high frequency bin in OA-treated hypoxic nerve cord was stabilized for the length of axon contained within the ganglion after 10 minutes of treatment such that MRCD did not change significantly throughout the remainder of the experiment (Figure 8ii-iv). MRCD for the high frequency bin continued to increase for axon within the untreated ganglion such that by 45 minutes and for the last analysis at 65 minutes there was a significant difference between treated and untreated ganglia (p < 0.001) (Figure 8iii, iv). Mean percent fidelity
of APs (MPFAPs) for both OA-treated hypoxic nerve cords and their control was 100% 5 minutes before treatment (Figure 9i). MPFAPs of OA treated nerve cords 5 minutes before treatment was not significantly different than any of the subsequent time points analyzed where as MPFAPs of untreated hypoxic nerve cords 5 minutes before treatment was significantly different than all subsequent time points analyzed (p < 0.001) (Figure 9i-iv). By 30 minutes and at 50 minutes of treatment MPFAPs for OA treated nerve cords was significantly different than that of its control (p < 0.001) and (p < 0.007) (Figure 9iii, iv). Mean maximum relative conduction delay (MMRCD) of OA treated nerve cords was significantly different than that of its hypoxic control by 30 minutes of treatment and at 50 minutes of treatment (p < 0.001) (Figure 9iii, iv).

Octopamine did not affect spike count as measured with electrode b from the prothoracic-mesothoracic connective (Figure 10i). Octopamine did not attenuate the reduction in peak frequency of hypoxic nerve cords relative to their normoxic control and there was no difference between OA-treated hypoxic nerve cords and their control for any of the times analyzed (Figure 10ii).

_Bath applications of metformin or ZD7288 compromise spike train propagation in preparations with normoxic nerve cords_

Individual responses to the looming stimulus in normoxic nerve cords were stable over long periods of time (Figure 11i). Applications of either 10 mM metformin or 100 μM ZD7288 compromised spike train propagation by simultaneously increasing the
Figure 11. Individual responses to loom during the bath application and washout of metformin and ZD7288; conduction delays plotted against their respective instantaneous frequency. i) Control experiment; trachea intact (delay from electrode b to c). ii) Bath application of 10mM meformin at 26 minutes followed by washout at 46 minutes (delay from electrode b to c). iii) Bath application of 100 mM ZD7288 at 26 minutes followed by washout at 46 minutes (delay from electrode a to c).
conduction delays and reducing the frequency range over which the peak firing rate of the spike train occurred (Figure 11ii, iii). The effects of metformin and ZD7288 on spike train propagation were also effectively reversed with sufficient washing out of the preparation.

In normoxic nerve cords receiving no treatment there was no difference within in low or medium frequency bins of mean relative conduction delay (MRCD) before (25 minutes) during (45 minutes) or after treatment (55 minutes), however in the high frequency bin there were differences in MRCD before-to-during, during-to-after (p = 0.009) and before-to-after treatment (p < 0.001) (Figure 12i). In normoxic nerve cords receiving a treatment of 10 mM metformin between 26 and 46 minutes there was a difference in MRCD for the low frequency bin before-to-during treatment (p < 0.001) (Figure 12ii). Within the medium frequency bin there were differences in MRCD before-to-during (p < 0.001) and during-to-after treatment (p = 0.008) (Figure 12ii). Within the high frequency bin there were differences in MRCD before-to-during (p < 0.001), during-to-after (p = 0.002) and before-to-after treatment (Figure 12ii). In normoxic nerve cords receiving a treatment of 100 μM ZD7288 between 26 and 46 minutes there were differences in MRCD for the low frequency bin before-to-during treatment (p < 0.001) and during-to-after treatment (Figure 12iii). Within the medium frequency bin there were differences in MRCD before-to-during treatment (p < 0.001) and during-to-after treatment (p = 0.003) (Figure 12iii). Within the high frequency bin there were differences in MRCD before-to-during treatment (p < 0.001) and during-to-after treatment (p = 0.003) (Figure 12iii).
Figure 12. Conduction velocity in the DCMD axon was reversibly decreased by pharmacological manipulations with Metformin or ZD7288 in normoxic nerve cords. Mean relative conduction delay (MRCD) was determined for control (normoxic), experimental group 1 (normoxic/metformin) and experimental group 2 (normoxic/ZD7288) before, during and after drug application i.e. washout. MRCD was determined using the delays recorded between electrodes b and c. Under control conditions (i) there is an increase in conduction delay for the high frequency bin and an increase in conduction delay over time within the high frequency bin. In both the metformin (ii) and ZD7288 (iii) treatments there is a during treatment effect for all three frequency bins (p < 0.001) indicated by asterisks (*). There is a complete washout for each frequency bin of ZD7288 (iii) and a complete washout for the medium frequency bin of metformin (ii).
Comparing the low frequency bins of MRCD in normoxic control, metformin treated and ZD7288 treated groups there were no differences between groups before or after treatment. During treatment, however, there were significant differences between normoxic control and each treatment group (p < 0.001) (Figure 12i-iii). During treatment there was also a difference between the metformin and ZD7288 treated groups. Comparing the medium frequency bin of MRCD there were no difference between any of the groups control or treated before treatment (Figure 12i-iii). During treatment there were differences between the control group and each of the treatment groups (p < 0.001) but not between treated groups (Figure 12i-iii). After treatment there was only a difference between the control and metformin-treated groups (Figure 12i, ii). Comparing the high frequency bin there were no differences between any of the groups control or treated before or after treatment (Figure 12i-iii). During treatment there were differences between the control group and each of the treatment groups (p < 0.001) but not between treated groups (Figure 12i-iii).

There was no difference in spike count from before-to-during, during-to-after or before-to-after treatment for the normoxic control, metformin and ZD7288 treated groups (Figure 13i). There was no difference in peak frequency before-to-during, during-to-after or before-to-after treatment for the normoxic control (Figure 13ii). In the metformin-treated group there was a difference in peak frequency before-to-during (p = 0.002) and during-to-after (p < 0.001) but not before-to-after treatment (Figure 13ii). In the ZD7288 treated group there was a difference in peak frequency before-to-during and during-to-after (p < 0.001) but not before-to-after treatment (Figure 13ii).
Figure 13. Peak frequency in the DCMD axon was reversibly decreased by pharmacological manipulations with metformin or ZD7288 in normoxic nerve cords and spike count was unaffected. Peak frequency and spike count were determined for control (normoxic), experimental group 1 (normoxic/metformin) and experimental group 2 (normoxic/ZD7288) before, during and after drug application i.e. washout. Spike count and peak frequency were determined from electrode b. i) Neither treatments with metformin or ZD7288 had an effect on spike count. ii) Both treatments with Metformin and with ZD7288 reversibly reduced the peak frequency recorded at electrode b.
*Metformin and ZD7288 affect the amplitude, duration and shape of action potentials as interpreted from their triphasic waveforms recorded extracellularly*

Triphasic extracellular recordings can be used to infer the monophasic action potential since the negative peak of the triphasic action potential has been shown to correspond to within 0.04 ms of the peak membrane action potential (Pearson *et al.* 1970). An overlay of three action potentials, each having the lowest instantaneous frequency within spike trains generated before, during and after treatment with metformin, demonstrates a reversible change in triphasic waveform recorded extracellularly from electrode b (Figure 14i). A similarly generated overlay before, during and after treatment with ZD7288 demonstrates the same changes in the triphasic waveform (Figure 14ii). In both overlays the general trend was a decrease in amplitude in each of the three phases of the triphasic recording and an increase in the duration of the action potential.

Both metformin and ZD7288 reversibly increased the duration of low frequency action potentials as measured from the positive peak of the first phase to the positive peak of the third phase of the triphasic recording. In normoxic nerve cords treated with metformin there were differences before-to-during (p = 0.001) and during-to-after treatment (p = 0.015) (Figure 15ai). In normoxic nerve cords treated with ZD7288 there were differences before-to-during (p < 0.001) and during-to-after treatment (p = 0.015) (Figure 15a(ii)). There were no differences between metformin or ZD7288 treatments before, during or after treatment.
Figure 14. Overlays of the triphasic waveforms for action potentials generated before during and after pharmacological manipulation. Overlays show 0.25 ms before and 0.75 ms after the peak of the first phase of the triphasic waveform for the AP of the lowest instantaneous frequency within a spike train recorded from electrode b. The change in AP waveform generated by treatments with either metformin or ZD7288 are largely in the second half of the second phase of the triphasic which corresponds to the downstroke of the monophasic action potential.
Figure 15. Pharmacological manipulations of duration and amplitude during the second and third phases of the triphasic waveform. Time and voltage coordinates for the peak positive voltage of the 1st (yellow) and 3rd (blue) phases and the peak negative voltage of the 2nd phase (green) were determined for the AP of the lowest instantaneous frequency within a spike train recorded from electrode b. The coordinates were used to determine peak to peak durations and amplitudes as well as the amplitudes of the 1st and 3rd phases. a) Metformin and ZD7288 reversibly increase the AP duration (i, ii) the largest component of which is the second half of the second phase and the first half of the third phase (iii, iv). b) The amplitude between the peak of the second phase and the peak of the third phase is reduced during treatments with either metformin or ZD7288 (i, ii) and the amplitude of the third phase is similarly reduced (iii, iv).
Both metformin and ZD7288 reversibly increased the duration of low frequency action potentials as measured from the negative peak of the second phase to the positive peak of the third phase of the triphasic recording. In normoxic nerve cords treated with metformin there were differences before-to-during (p = 0.008) and during-to-after treatment (p = 0.019) (Figure 15aiii). In normoxic nerve cords treated with ZD7288 there were differences before-to-during (p < 0.001) and during-to-after treatment (p = 0.002) (Figure 15aiv). Treatment with ZD7288 produced a significantly greater increase in AP duration recorded from the negative peak of the second phase to the positive peak of the third phase (Figure 15aiii, iv).

Metformin reversibly decreased the amplitude of the action potential as measured from the negative peak of the second phase to the positive peak of the third phase of the triphasic recording. In normoxic nerve cords treated with metformin there were differences before-to-during and during-to-after treatment (p < 0.001) (Figure 15bi). ZD7288 produced a decrease in the amplitude of the action potential as measured from the negative peak of the second phase to the positive peak of the third phase of the triphasic recording. In normoxic nerve cords treated with ZD7288 there was a difference before-to-during treatment (p = 0.001) (Figure 15bii) however, the effect of ZD7288 was not removed completely by its washout. Metformin and ZD7288 also appeared to affect the amplitude of the third phase of the triphasic recording relative to a baseline of 0 mV (Figure 15biii, iv). Metformin reversibly decreased the amplitude of the first phase of the triphasic recording. In normoxic nerve cords treated with metformin there are differences before-to-during and during-to-after treatment (p < 0.001) (Figure 16i).
Figure 16. Metformin reversibly affects the amplitude of the first phase in the triphasic waveform. The peak positive voltage of the 1st (yellow) phase was determined for the AP of the lowest instantaneous frequency within a spike train recorded from electrode b. Metformin was found to decrease the amplitude of the first phase.
ZD7288 also appeared to affect the amplitude of the first phase of the triphasic recording relative to a baseline of 0 mV (Figure 16ii).

Two dimensional raster plots of spike trains before, during and after treatment with either metformin or ZD7288 demonstrate the wide spread effect of these drugs on individual action potentials throughout the entirety of the spike train (Figure 17i, ii). The largest effect of both metformin and ZD7288 appeared to be during the second half of the triphasic recording which corresponds to the hyper-polarization phase of the action potential. An increase in the duration of the hyper-polarization phase can be easily seen as a broadening of separate colour bands within the colour spectrum from 1 ms to 1.5 ms. The duration and location of the red band at 1.5 ms increases and shifted to the right during treatments with metformin or ZD7288, indicating a shift in the third phase of the triphasic recording. A broadening of the red band at 1 ms and its shift toward the blue end of the colour spectrum was more apparent during metformin treatment than ZD7288 treatment and indicates an increased duration and decreased amplitude.

*Non-uniform additive increases in conduction delay over the entire length of DCMD axon are significant in terms of arrival time and may result in the corruption of temporally encoded information*

In normoxic nerve cords the delay of individual action potentials was dependent on their instantaneous frequency. The action potential having the lowest instantaneous
Figure 17. 2-D raster plots of all the APs within a spike train show that the triphasic waveforms for APs of various instantaneous frequencies are affected by pharmacological manipulations with either metformin or ZD7288.
Figure 18. Multi-electrode triphasic action potential overlays. The same three seconds from 2-3 separate electrodes are lined up with respect to the action potential in the most anterior electrode. a) The action potential with the highest instantaneous frequency (labeled red) displays the highest delay in all cases shown. b) The triphasic waveform of action potentials as seen in electrode a for metformin (ii) and ZD7288 (iii) treatments change from before-to-during and during-to-after. c) delays are additive down the length of the axon as seen when comparing electrodes b and c to electrode a (ii and iii). d) Action potentials of low instantaneous frequency display similar conduction delays whereas APs of high instantaneous frequency display a large variation in conduction delay.
the highest instantaneous frequency had the largest conduction delay between recording electrodes (Figure 18i). Comparisons of the additive delay observed between closely placed electrodes (2 mm = a to b) and electrodes placed further from one another (4.5 mm = a to c) revealed that delays were additive and that the differences in conduction delay between low and high frequency APs increased with increased distance of propagation (Figure 18ii, iii). Thus, APs of high instantaneous frequency moved further from the AP that preceded it while moving closer to the AP it proceeded. Application of metformin or ZD7288 simultaneously altered the wave form of the triphasic recording and increased the propagation delay between recording electrodes (Figure 18ii, iii).

When considering the additive delay over the entire length of axon before, during and after treatment with either metformin or ZD7288 a small increase in delay across electrodes spaced a few millimeters apart add up to an increase that is on a time scale which may be physiologically significant. Considered separately, both metformin and ZD7288 increase the total delay between LGMD and FETi within each of the frequency bins (p < 0.001) (Figure 19i, ii). Wash out of the drugs significantly reduces the total delay within each of the frequency bins considered compared to the total delay during treatment (p < 0.001) (Figure 19i, ii). A successful wash out was only observed for the low frequency bin of metformin treated nerve cords, in all other cases the total delay before treatment was significantly different than the total delay after treatment (p < 0.001) (Figure 19i).
Figure 19. Interpreting the cumulative delay in DCMD axon incurred between the (source) LGMD and (destination) FETi. The time taken for the transmission of action potentials between the LGMD and the FETi was estimated by using rough measurements of the total distance between the two along the DCMD axon as well as the spacing of recording electrodes. Pharmacological treatments of either metformin or ZD7288 increased the time course of propagation from source to destination. The time course was reduced following a washout of the drugs however, the time course was only fully restored for the low frequency bin of action potentials treated with metformin.
The shift in relative timing of spikes within a spike train, not as a function of length but as a function of drug treatment, can be demonstrated with an inter-spike-interval (ISI) profile generated from numerous spike trains recorded from the same location along the length of DCMD axon before during and after treatment. Both metformin and ZD7288 reversibly decreased the number of events with ISI ($3 \leq x < 4$ ms) and reversibly increase the number of events with ISI ($4 \leq x < 5$ ms) Figure 20. (ii, iii). The ISI profile for the normoxic control did not change over the course of the experiment (Figure 20i).
Figure 20. Pharmacological manipulation of inter-spike-intervals. Using recordings from electrode b, histograms made with a resolution of 1 ms. Unlike i) the normoxic control ii) metformin or iii) ZD7288 treated normoxic nerve cords show a reversible shift between the number of events with inter-spike-intervals in the 3 ms (333-250Hz) and 4 ms (250-200Hz) bins from before-to-during and during-to-after treatments with either (arrows indicate shifts).
CHAPTER 4: DISCUSSION

Oxygen is of critical importance in the generation of the ATP necessary for the optimal function of cells. It is also, however, the source of reactive oxygen species which damage the bio-molecules necessary for optimal function. Therefore it is important that animals have a system of delivery, which acts to regulate the amount of oxygen reaching sensitive tissues within an appropriate range. Insects possess a tracheal system which functions to exchange gases between the atmosphere and tissues. Air enters the tracheal system through a set of spiracles, which possesses valves that can be opened or closed. Tracheae branch into progressively smaller and finer tracheoles until they reach a size at which $O_2$ can diffuse across the tracheolar membrane into the cells various tissues. When oxygen is limiting insects can actively increase the rate of ventilation in an attempt to maintain an adequate supply of oxygen to respiring tissues. When tissues have a reduced respiratory demand insects can conversely decrease or arrest ventilation and even close spiracular openings to avoid the unnecessary production of reactive oxygen species.

Insects respond to hypoxia by making compensatory changes to their spiracular openings as well as changing the extent to which they actively ventilate (Harrison et al. 2006). When these compensatory changes are insufficient, cells have two strategies available to them in order to restore the balance between supply and demand of ATP. The cells can either increase ATP supplies via anaerobic pathways or reduce demand (Hochachka 1986). Increasing ATP supply through substrate level phosphorylation, however, is wasteful of substrate and shortens survival time in an atmosphere of reduced
oxygen. Alternatively, reducing ATP demand serves to increase survival time in reduced oxygen (Bickler and Buck 2007). Hochachka (1986) coined the term generalized “channel arrest” to describe a decrease in cell membrane permeability in order to account for the large scale drop in absolute Na⁺/K⁺ ATPase activity and the concomitant maintenance of normal electrochemical gradients. In neurons, the most energetically expensive activity is re-establishing ion gradients following an action potential. The down regulation of firing rates and or synaptic transmission during hypoxia/anoxia was termed “spike arrest” by Hochachka et al. (1993) and is thought to contribute the most energetic savings in spiking neurons.

Down regulation of firing rates and reduced permeability of membranes at rest is a means for the continued maintenance of ionic gradients across the plasma membrane in neurons. When there is insufficient energy to actively pump ions against their thermodynamic equilibrium, the strategy of down regulating the demand on Na⁺/K⁺ ATPase in favour of long-term stability of ionic gradients comes at the cost of neuronal performance. The goal of this thesis was to characterize the performance trade-off that occurs in a visual neuron of the orthopteran L. migratoria when the neuron is exposed to hypoxic conditions. An attempt was also made to identify conductances critical to measures of performance that may be down-regulated in response to hypoxia and potential pathways that mediate changes in membrane permeability/performance.

Axonal performance of the DCMD was significantly diminished during hypoxia. Conduction delay of APs increased progressively over the course of a 65-minute experiment in which DCMD axon was subject to hypoxic conditions. Propagation of high
frequency APs was affected preferentially. Relative spike timing within the spike train was also compromised, and was readily seen as the difference in conduction delay between frequency bins. These effects became increasingly apparent during the course of the experiment. Under hypoxic conditions frequency dependent failures occurred within the mesothoracic ganglion. There was, however, no reduction in the spike-count of spike trains generated over the course of the experiment, suggesting that the site of spike train initiation in the DCMD, namely the protocerebrum, remained normoxic over the course of the experiment. When the conditions experienced by DCMD axon in the nerve cord were normoxic, conduction velocity of APs remained stable over the course of the 65-minute experiment and the influence of instantaneous frequency on AP delays was minimal. An estimate of conduction velocity for the DCMD axon was made for both the normoxic nerve cord and hypoxic nerve cord, indicating a 54% reduction in conduction velocity for axon in the hypoxic nerve cord. There was a similarly large reduction in the peak instantaneous frequency recorded from the hypoxic nerve cord when compared with the normoxic nerve cord. Strikingly, no AP failures were observed within the mesothoracic ganglion under normoxic conditions, suggesting that under normoxic conditions branch points do not display a reduced safety factor. Application of 0.1 mM octopamine 15 minutes into the 65 min experiment attenuated the effects of hypoxia on conduction velocity, delay profiles of frequency bins and AP failures within the mesothoracic ganglion.

The selective blocker of HCN channels ZD7288 and the AMPK activator metformin both markedly reduced conduction velocity in DCMD axon. Neither drug had
an effect on spike count nor did they produce any AP failures within the mesothoracic ganglion. However, they did reduce peak frequency of spike trains and both drugs affected AP durations and amplitudes of the triphasic waveform recorded extracellularly.

Taken together these results suggest that under hypoxic conditions there is reduced neuronal performance in the DCMD axon with respect to timely and accurate transmission of spike trains. The data indicate that HCN channels are required for the timely and accurate transmission of spike trains under normoxic conditions and reduced performance of DCMD axon under hypoxic conditions may be the result of HCN down regulation during hypoxia. Since the AMPK pathway activator metformin produced strikingly similar effects to the HCN selective inhibitor ZD7288, metformin may be affecting HCN current through an unknown mechanism. Both drugs arguably affect sodium channels, as they reduce both the conduction velocity and the peak frequency of spike trains. Just how sodium channels might be affected under each of the above mentioned experimental conditions is however, not entirely clear.

*Axonal conduction velocities observed under normoxic conditions are not supported by DCMD during hypoxia*

In non-myelinated axons, conduction velocity depends on several biophysical factors. In a recent review on axon physiology Debanne et al. (2011) outlined a few of the principle factors influencing conduction velocity which included; the number of sodium channels available, membrane capacitance, internal impedance and temperature.
The more Na\(^+\) channels available when the membrane reaches its voltage threshold the steeper the upstroke of the action potential, making the spatial voltage gradient steeper between the fully depolarized patch of membrane and the adjacent patch of membrane yet to be disturbed by the invading Na\(^+\) current. The result of having increased channel availability is that the excitation of adjacent membrane is faster, allowing the AP to propagate with an increased conduction velocity. A higher membrane capacitance or a larger amount of charge stored on the membrane per unit area reduces the amount of time necessary to reach threshold. High intracellular ion mobility in large diameter axons results in low internal impedance of Na\(^+\) current into the cell and likewise reduces the time necessary to reach threshold. The speed of Na\(^+\) current spread on the inside of the membrane adjacent to that of the membrane experiencing the upstroke of the AP dictates the speed of propagation. This spreading of Na\(^+\) current is dependent on any number of variables, only a few of which were addressed by Debanne and colleagues (2011).

A previous estimate of conduction velocity in DCMD axon made by O’Shea et al. (1974) of 3.1 m/s identical to that of the estimate made here of 3.1 m/s using conduction delays recorded from DCMD axon contained within hypoxic nerve cords. However, the estimate of conduction velocity made here of 5.7 m/s for DCMD axon under aforementioned normoxic conditions is likely a more accurate estimate of the true conduction velocity for this axon in an un-dissected animal. Perhaps not surprisingly, Bickler and Buck (1998) point out that the preservation of critical ion gradients by down regulating Na\(^+\) ion channel activity should result in decreased nerve conduction velocity as well as an elevated action potential threshold. The reduced conduction velocity of
DCMD axon in the hypoxic nerve cord observed here is most certainly owing to a reduction of Na\(^+\) current, the exact cause of the reduction in sodium current is however, far more uncertain.

Some possible mechanisms for reducing Na\(^+\) channel activity in response to hypoxia include; channel trafficking, protein phosphorylation and modulation of the resting potential thereby reducing the number of Na\(^+\) channels available when the membrane reaches threshold potential. Constitutive channel trafficking of voltage gated sodium channels, though metabolically costly, may permit a change in membrane channel density on a relatively short time scale (Fortune and Chacron 2009). In the context of this thesis a change in membrane channel density may act to stabilize ionic gradients across the membrane, reducing the total Na\(^+\) channel activity by virtue of reducing the number of channels expressed in the membrane. Indeed, reducing Na\(^+\) channel activity with the application of tetrodotoxin has been shown to reduce hypoxic-induced neuronal injury and death both \textit{in vitro} and \textit{in vivo} (Weber and Taylor 1994; Prenen \textit{et al}. 1988). Another way in which hypoxia may induce Na\(^+\) channel inhibition advocated by O’Reilly \textit{et al}. (1997) is through protein phosphorylation. The number of Na\(^+\) channels available for activation at a given resting potential is thought to be reduced by producing a negative shift in the steady-state inactivation of Na\(^+\) channels. Rather than changing the voltage properties of the Na\(^+\) channels themselves, it is possible that merely changing the resting membrane potential of axonal membrane may reduce the number of channels whose current is effective in recruiting adjacent channels.
The hyperpolarization-activated current ($I_h$) of the HCN channels has been shown to contribute to the resting potential of neurons (Doan and Kunze 1999). $I_h$ current has been shown to be inhibited by hypoxia, which shifts HCN activation potential to a more hyperpolarized level in less than three minutes in thin axon and results in membrane hyperpolarization (Gao et al. 2006). $I_h$ current is also blocked by the application of ZD288 a selective blocker of the HCN channel, which reduces axonal conduction velocity in cerebellar parallel fibers and resulted in conduction failures at branch points (Baginskas et al. 2009). There is strong evidence for the expression of HCN in locust octopamine dorsal unpaired median neurons, indicated from their electrophysiological profile obtained from both voltage- and current-clamp experiments in response to ZD7288, Cs$^+$, Ba$^+$ and TEA$^+$ (Heidel and Pfluger 2006). Blocking HCN channels likely hyperpolarizes the resting potential of DCMD axonal membrane increasing the number of closed sodium channels at the resting potential. While more sodium channels are available for depolarization, a greater amount of the finite sodium current is required to bring the membrane to threshold, increasing the time course of propagation and reducing the amount of current available for subsequent depolarization’s of adjacent membrane.

Whereas membrane potentials cannot be inferred from extracellular recordings, when comparing DCMD axonal performance under normoxic and hypoxic conditions similar changes occur that are seen in axons of other neurons when HCN current is blocked. Conduction velocity was severely diminished for DCMD axon in hypoxic nerve cord whose earliest recorded velocity was nearly half of that recorded from DCMD axon in a normoxic nerve cord. However, the reduction in conduction velocity continued over
the first 45 minutes of recording. When the time between the placement of the metal plate beneath the ganglia and the time of the first recording are considered, the time course of conduction velocity decrease is reminiscent of the time course of HCN rundown reported by DiFranesco et al. (1986), approximately 65 minutes.

While branch point failures were observed in the DCMD axon in response to hypoxia, no branch point failures were seen under normoxic conditions after the application of ZD7288. A computational study of branch points by Goldfinger (1999) indicates that conduction velocity changes, as a function of spatial inhomogeneities and AP propagation is in fact reliable across branch points as well as increases in axonal diameter. It has been suggested that geometrical constraints cannot fully account for conduction failures and that inactivation of sodium channels by high levels of $[\text{K}^+]_o$ as a consequence of repetitive firing increases the proportion of conduction failures (Debanne et al. 2004: Debanne 2011). The reason AP failures were only observed in hypoxic preparations may have to do with the fact that the ability to buffer $[\text{K}^+]_o$ at branch points or simply within the mesothoracic ganglion was compromised in hypoxic but not in normoxic nerve cords.

Octopamine was found to attenuate the time course of reduced performance both in terms of conduction velocity and conduction failures at branch points. Perhaps this is owing to the ability of OA to activate adenylate cyclase in the locust as demonstrated by Armstrong et al. (2006). It is possible that increasing cAMP via octopamine application might shift the activation potential of HCN channels to a more depolarized level and secure some of its depolarizing activity at resting potentials, resulting in the attenuation
of hypoxic effects on conduction velocity. It is also possible that OA application leads to a reduction in \([K^+]_o\) accumulation through a buffering mechanism, thereby reducing the number of AP failures. Octopamine has been previously shown to reduce the permeability of cockroach perineurium to \(K^+\) moving across the blood brain barrier from the haemocoel into the nerve cord (Schofield and Treherne 1985). Experiments where octopamine treatment begins either before or at the onset of hypoxia may act simply to stabilize rather than decrease conduction delays. Perhaps the most novel and thus most intriguing finding in this thesis is the finding that the anti-diabetic medication and AMPK pathway activator, metformin, was equally capable as ZD7288 of modifying axonal conduction velocity in DCMD axon under normoxic conditions. Possible mechanisms will be considered in the final section of the discussion.

As outlined earlier in this thesis, the DCMD axon makes a large number of direct and indirect synaptic connections to motor circuits throughout the mesothoracic and metathoracic ganglia. These connections are likely required for a variety of complex behaviours. It is therefore important that the DCMD excite its postsynaptic neurons in a synchronous manner appropriate to the production of adaptive behaviours. The idea of synchronized spatial firing of downstream neurons was expressed most eloquently by Chung et al. (1970) whose proposition was later quoted by Segev and Scheidman (1999) and bears repeating here: “The axonal arborization acts to transform the temporal pulse patterns of the parent axon into spatial patterns in its terminals… Thus, at the outset, we are confronted not with a system having only two stages at the output, on and off, but with one having a large number of possible combinations of active and inactive
terminals”. Taking this idea a step further, the DCMD is not the sole input onto
downstream target neurons. Thus a second level of synchrony with other visual non-
DCMD inputs onto downstream neurons highlights the importance of DCMD conduction
speed. For review of the proposed circuitry involved in producing a locust jump,
including contributions from six descending movement detectors, several modalities,
proprioreceptive feedback and potential gating mechanisms see Fotowat and Gabbiani
(2011).

The effect of reduced conduction velocity on the behavioural performance of the
locust during escape jumping will require a similar technique as the one employed by
Fotowat et al. (2011) where recordings were made from freely moving animals. It might
then be possible to determine the effect of reduced conduction velocity as a result of
hypoxia, treatment with ZD7288 or metformin and whether OA acts to maintain adaptive
behaviour during hypoxia.

DCMD axon fails to support the propagation of high frequency action potentials during
hypoxia: Consequences for temporal coding

Experimental evidence supporting the idea that axons are more than simply
passive delay lines has been accumulating for more than a decade. The importance of the
role axons play in the transformation of signals during their propagation between their
point of origin, the axon hillock, and their destination, the synapse, is demonstrated by a
recent wealth of reviews (Segev and Scheidman 1999; Debanne 2004; Kress and
Mennerick 2009; and Debanne et al. 2011). Perhaps the most fundamental question to be asked about faithful AP propagation in the axon is whether or not there is a surplus with regard to the density of sodium channels in the axonal membrane. Madeja (2000) addressed this very question by gradually increasing the concentration of TTX over a series of experiments and was able to show that only one third of the Na⁺ channels in the axonal membrane were necessary for the generation of an individual AP. However, a surplus of Na⁺ channels was necessary for repetitive action potential generation. Madeja (2000) was able to show that the purpose of a high density of Na⁺ channels fulfils the role of repetitive firing as opposed to the role of mere AP generation. It is likely that had it been investigated here, sodium channel density would also have played a role in conduction velocity. The investigation of Madeja (2000) into Na⁺ channel density has shown that the ability of neuronal membrane to support repetitive firing is not mutually exclusive from the ability of axonal membrane to propagate APs at high velocity.

As pointed out by Kress and Mennerick (2009), it is not only the fidelity and the timing, but also the waveform of APs during propagation, that effect synaptic communication (adaptive behaviour). They are critical determinants of synchrony (a function of conduction velocity) and efficacy (a function of spike frequency). In the same paper reporting a conduction velocity of 3.1 m/s for DCMD axon, Burrows and Rowell (1973) also report that excitatory postsynaptic potentials were elicited in the fast extensor tibiae (FETi) by DCMD frequencies greater than 200 Hz. However, depolarization of the soma never exceeded 4 mV and spikes were not evoked in FETi. It is likely that the frequency of 200 Hz was near the peak frequency observed by Burrows and Rowell, as
they would have likely reported a peak frequency if it were significantly higher than 200 Hz. Under normoxic conditions I regularly observed instantaneous frequencies approaching 500 Hz and in some cases frequencies as high as 600 Hz, which may well have elicited spikes in the FETi however, recordings from FETi during high frequency stimulation are needed to confirm this suspicion. The peak frequencies I observed under normoxic conditions were in stark contrast with those I observed under hypoxic conditions, when the frequency rarely exceeded 350 Hz and was often much lower.

It is well established that sensory neurons transmit information about external stimuli using trains of APs, however the unit of information contained within spike trains is little understood. A few of the coding schemes currently considered include rate codes, temporal codes and multiplexed codes. The idea that the firing rate contains information about a stimuli originated from the work of Adrian and Zotterman (1926) who demonstrated that the firing rate of a stretch receptor in muscle is related to the force applied to the muscle and hence the stimuli strength experienced by the stretch receptor. Rate coding averages the number of spikes within a given window of time however, neglects the temporal pattern of spikes within a given window of time.

Temporal coding is concerned with the pattern of spikes within given windows of time where the relative timing of spikes carries important information about the stimulus. VanRullen et al. (2005) point out that the time course of behavioural responses to stimuli are too short for sensory processing of neuronal firing rates over extended windows of time and that temporal codes account for rapid behavioural responses. Evidence for the importance of precise spike timing exists for a diversity of modalities: visual neurons in
the cat (Reinagel and Reid 2000), somatosensory neurons in humans and rats (VanRullen et al. 2005; Panzeri 2001) and auditory neurons of a number of species including the locust (Rokem et al. 2006).

Yet another sensory code using multiplexed temporal scales within the same spike train have been suggested and argued to increase the amount of information available within a spike train. When considering a multiplexed code, a single spike train may encode both the contrast of a visual stimuli using the temporal precision of ~10 ms and orientation or spatial frequency of the same stimuli using a coarser scale of 30-100 ms (Panzeri 2009). A recent computational study compared the behavioural performance of an animal during a visual task with the information available to the animal in terms of spike trains recoded from the optic nerve when the retina is stimulated with the same visual stimuli. The information assumed available to the animal depended on the coding scheme used and it was found that whereas coarse spike count codes could not account for behavioural performance of the animal, a finer temporal correlation code rich in information could account for the animals behaviour (Jacobs et al. 2009).

It is important to remember that the AP frequency measured from the DCMD is generated by the LGMD located in the third neuropil of the optic lobe. Therefore, the peak frequency recorded from the DCMD axon is dependent on the initial frequency set in the brain. That being said, having access to multiple recordings along the length of the DCMD axon reveals that delays are additive along the length of the DCMD such that the peak frequency is steadily diminished as the spike train propagates down the length of the DCMD. If we consider three action potentials in a row the first of low instantaneous
frequency followed immediately by a second action potential (high instantaneous frequency), which is followed closely by a third AP of low instantaneous frequency. As the three APs propagate the first and third AP will move at a relatively constant velocity, whereas the second AP will slow down changing not only its instantaneous frequency but that of the third action potential whose instantaneous frequency is thus increased. The reliable and accurate transmission of spike trains is dependent on axonal propagation, which sets a limit on the amount of information a spike train can encode and transmit. Schneidman and Segev (1998) compare stochastic and deterministic Hodgkin-Huxley models and demonstrate that in fact the stochastic nature of ion channels may have significant effects on the reliability and accuracy of spike trains in spite of a high density of excitable channels. They show that jitter occurs to a greater extent in response to direct current compared with white noise. In a later paper the same authors try to account for spike jitter. They suggest that near the threshold potential, only a small percentage of ion channels are available and that a large variability in this small number of open channels near threshold introduces a “jitteriness” into the spike firing time (Segev and Schneidman 1999).

Although Segev and Schneidman were referring to the spike initiation zone, the nature of excitable channels, namely the number of open Na⁺ channels near threshold at a given point along the axon, may constitute the same repercussions in terms of a jittery propagation. If the number of open channels at any given point along the axon is highly variable the consequences for accurate propagation of the temporal nature of a spike train are rather extreme. Faisal and Laughlin (2007) investigated spike-timing jitter in thin
axons suggesting that spike-timing is jittered through both the variability in the number of Na+ channels available during the early rising phase and “stochastic microsaltatory conduction” whereby an AP can leap over a patch of membrane where no Na+ channels are open to an area where a sufficient number of Na+ channels are open. HCN channel activity is also important for processing. $I_h$ current generated through the HCN channels limits the range of hyperpolarizations and/or depolarizations produced by the accumulation of external and/or internal accumulation of ions (Debanne 2004).

When cAMP is bound to the HCN channel and $I_h$ current is activated at more positive potentials, $I_h$ current may act to stabilize the membrane potential in between high frequency spikes ensuring that the number of sodium channels available near threshold is both stable and sufficient for propagation of high frequency APs. When cAMP is limiting and the HCN channels are activated at more negative potentials, $I_h$ current may not be active over the time course necessary to stabilize the membrane potential in between high frequency action potentials and thus the number of available Na+ channels may prove to be insufficient. $I_h$ current has been shown to be important for the temporal processing of input patterns in neurons from the inferior colliculus of Wistar rats (Koch and Grothe 2003). It is possible that $I_h$ current may be important for the faithful propagation of temporal patterns in the axons of various neurons.

Although it isn’t formally addressed or analyzed here, propagation jitter was observed in both hypoxic and normoxic conditions for APs at or near the peak frequency of the spike train. However, because peak frequencies were different in normoxic and hypoxic conditions jitter was observed at ~300Hz in hypoxic preparation but not in
normoxic conditions where jitter was observed only at higher frequencies ~450Hz. Goldberg and Andreou (2004) considered the performance of rate code and temporal code in an inherently noisy channel considering the degrading effects of both spike jitteriness and spike deletions based on the ability of the spike train decoder to reconstruct the stimulus current. This was considered as an upper-limit on the amount of information available for computation. Golderberg and Andreou found that temporal decoders performed better than rate code when there was no channel noise (spike jitter or deletions). When channel noise consisted of either spike jitter or spike deletions, temporal decoders only outperformed rate decoders when stimulus reconstruction needed to be fast i.e. a “quick and dirty” estimate of the stimulus was preferable to a “slow clean” estimate. Golderberg and Andreou also acknowledge that neurons typically make no attempt to reconstruct stimuli, instead they perform a rich array of computations on information generated from the original stimuli from one neuron to the next. In the case of the DCMD (channel) and FETi (decoder) under hypoxic conditions both the coarse nature (peak frequency) and the fine temporal nature (ISI) of the spike train are compromised, so that the supposed precisely encoded timing of FETi firing is compromised. Perhaps most importantly regardless of delayed arrival times, once the information has reaches the synapse the efficacy of the signal is likely compromised.

The propagation of a train of action potentials is useful only in so far as it is able to elicit its intended function post-synaptically. Due to probabilistic mechanisms of synaptic release at the synapse, transmission failures often occur (Allen and Stevens 1994). Single spikes or a series of spikes with insufficient temporal frequency may prove
unable to facilitate appropriate neurotransmitter release from the pre-synaptic cleft to properly excite the post-synaptic neuron (Thompson 1997). Bursts of high-frequency APs (<25 ms) help to ensure presynaptic facilitation and increase the probability of synaptic transmission (Lisman 1997). More recently Aldworth et al. (2011) investigated the temporal coding scheme used by single giant interneurons in the crickets cercal sensory system and found that the coding capacity of these neurons far exceeded expectations, due to their brief, highly precise patterns of APs. The behavioural relevance of bursts has also been demonstrated in the cricket where they have been shown to accurately detect salient increases in amplitude of ultrasonic sounds, which reliably signal the stimulus location and have allowed experimenters to then predict the behavioural response of the animal (Marsat and Pollack 2006). The ability to fire bursts of APs can be represented by a histogram of ISI’s. Using recordings from the DCMD, I was able to show that application of the HCN blocker ZD7288 or the AMPK activator metformin to a normoxic preparation reduced the ability of the axon to propagate high frequency bursts of APs. A reduced ability to fire high frequency APs may have an adaptive function in terms of an increased hypoxia tolerance of the DCMD axon, however, hypoxia tolerance in this sense is most likely a trade-off with performance, but further investigation is needed.

Characterizing the changes in performance that occur during the transition between normoxic and hypoxic conditions as they relate to behaviour of the animal could prove to be useful. Perhaps using a wireless telemetry system similar to Fotowat and Gabbiani (2011) and recording from the same animal under normoxic and hypoxic conditions might help to understand the features of the neural code necessary to elicit
adaptive behaviour. Other pharmacological experiments are needed confirm the presumed exclusive action of ZD7288 on HCN channels and axonal performance. Typically, confirmation of HCN current manipulation includes the separate applications of Cs\(^+\) ions, which also block HCN and Ba\(^+\) ions, which block the potassium inward rectifier (Kir) channels.

The mechanism by which metformin activates AMPK is unknown, and although pharmacological investigation into its effect on DCMD performance may prove to be complicated, it can be equally viewed as an opportunity to narrow down and identify a potential mechanism of action. Such investigations could add to a list of effects downstream to AMPK activation or possibly identifying an unknown mechanism of action upstream to the activation of AMPK. These will be considered at the end of the discussion.

*Reduced DCMD performance during hypoxia: A trade off resulting in maladaptive behaviour or an inherently adaptive strategy?*

The involvement of DCMD in mediating escape behaviours in the locust has been well characterized. In response to looming stimuli during flight the DCMD mediates a characteristic gliding dive (Santer *et al.* 2006) whereas on the ground it mediates a ballistic escape jump (Fotowat and Gabbiani 2011). I have shown that under hypoxic conditions axonal performance of DCMD is compromised such that the spike train encoded in the brain intended for motorneurons and interneurons in the thoracic ganglion
is degraded during its transmission along the DCMD axon. The consequences for the degradation of such a signal on first appraisal seem dire, however, they may in fact be of benefit to the animal.

As mentioned earlier in this thesis it is not always adaptive to elicit escape responses due to their highly stereotyped and thus highly predictable nature. In some cases opting for a “voluntary” response of long and variable latency rather than a short latency “reflexive” response mediated by giant fibers has been shown to be an adaptive strategy during dominant-subordinate encounters in crayfish (Krasne et al. 1997). Flush pursuers are a class of predatory bird that make use of their conspicuously patterned wings or tails to elicit escape responses in insects, driving their prey from a substrate and out of hiding directly into the predator’s line of sight. Flush-pursuers such as the painted redstart use foraging displays similar to simulated displays that trigger looming and time to collision neurons in escape circuits possessed by a variety of insects. The strategy employed by flush-pursuers consists of driving prey from a substrate such as a branch into the air where the flush-pursuer can then chase its prey, which in the air offers greater visual contrast and thus detection by its predator (Jablonski and Strausfeld 2000). It’s logical to think that a stereotyped response of this nature to a looming stimulus may become increasingly maladaptive when an animal is experiencing a metabolic stress. Flight is known to be the most energetically costly activity, capable of raising the metabolic rate 10-100 times its resting value (Rascon and Harrison 2005) and responding to flush-pursuers in this stereotyped fashion under metabolic stress would likely be a poor choice.
The alternative behaviour for the insect in response to a flush pursuit would be to hide or otherwise remain camouflaged and hope that the predator either doesn’t see them or makes a failed attempt at capturing them. Hassenstein and Hustert (1999) devised an experiment whereby they were able to characterize the hiding response of locusts in response to looming stimuli. They were able to show that the locust actively hid behind the vertical pole upon which it was perched, to varying degrees and at varying speeds. The animal’s response depended upon the lateral angle of approach and speed of approach of the looming stimulus presented on a 90 degrees angle with its eyes. Given that the hiding animal doesn’t have to resort to the metabolically expensive behaviour of flight, which would likely be of a sub-par performance for an animal experiencing metabolic stress, the hiding behaviour may have advantages that are twofold. The hiding animal may reduce its risk of predation (damage to the body superstructure) and the simultaneously reduce demand for high performance in the DCMD, which might otherwise lead to neuronal damage.

When the locust is at rest, the oxygen delivery capacity of its trachea is excessive. However when the locust is flying the safety margin for O₂ delivery is reduced (Rascon and Harrison 2005). Weis-Fogh (1967) eloquently demonstrated that abdominal pumping in the locust during flight passes air uni-directionally through giant tracheal tubes, with air moving in from spiracles 1-3 and out from spiracle 10. He showed that ventilation via abdominal pumping supplies the CNS where as thoracic pumping supplies flight muscles and that the locust refused to start flying if the first three pairs of spiracles were blocked with wax.
During the night spanning the dates of October 22\textsuperscript{nd} and 23\textsuperscript{rd} in 1978 an unusually dense concentration of grasshoppers was detected by radar in the Tilesmsi Valley in Mali and 2 hrs later, on a second radar 100 km south of its initial detection. Riley and Reynolds (1983) give a detailed account of this event, synthesizing data from the ground radar and the prevailing weather conditions of that night. Interestingly, the migration began at dusk and the peak elevation of this collective movement of animals reached 1300 m under the reduced illumination of starlight. Migrations of a sparser number of insects had been detected both before and after this night. What made the migration of this night so spectacular, as suggested by the investigators of this event, was the occurrence of a surge of dry northerly air creating a dramatic contrast with the previous nights storms and increasing the rate of take-off at dusk.

This account is of interest for several reasons. First, it could be argued not only that the grasshoppers are flying at night under reduced illumination to avoid detection by predators but also because of a reduced ability to detect predators. At an altitude of 1300 m the availability of O\textsubscript{2} is reduced and may have an effect on neurons sensitive to hypoxia such as the DCMD. A strategy of flying at night in large groups to avoid the need for high performance in DCMD may be necessary. Secondly, the suggestion of these investigators that the dry nature of the air may have stimulated the increased rate of take-off observed combined with the observation made by Weis-Fogh (1967) that locusts refused to start flying when the first three spiracles were blocked makes it difficult not to speculate that the air humidity may critically affect O\textsubscript{2} delivery during flight.
During data collection for this thesis on May 29th and 30th of 2011, the humidity in the Robertson lab in the Biosciences Complex at Queen’s University was comparable to that of the outside such that the cold pipes and taps in the building were covered in condensation. On these days, achieving a DCMD recording that wasn’t compromised over the period of 65 minutes in spite of several attempts (n = 4 /day) was not achieved. Saline was flowed through the animal such that the corkboard and surrounding air quickly became saturated with moisture, potentially blocking spiracles 1-3 and rendering the nerve cord hypoxic. While this is mere speculation, part of the procedure developed here for achieving stable high performance recordings included insuring that overflowing saline did not block spiracles. If the speculation here is correct it would be an interesting neuroethological constraint whereby locusts take advantage of the optimal conditions choosing to fly in dry air rather than humid air because they can’t support nervous function in humid air. It’s also worthwhile pointing out that the gliding dive in response to a looming stimulus only requires AP frequencies of \( >150 \text{ Hz} \) (Santer et al. 2006), suggesting that reduced performance during flight may be met with a preference for reduced firing rates by behaviourally relevant decoders (i.e. motorneurons relevant for flight behaviours).

**Mechanism of metformin action on DCMD axon**

The AMPK activator metformin and HCN channel blocker ZD7288 both affect spike train propagation in the DCMD axon in a similar way, reducing conduction velocity
and peak frequency such that the temporal nature of the spike train encoded in the brain is
distorted during its transmission to motor neurons in the thoracic ganglia. It is therefore
tempting to speculate that metformin may be exerting effects through HCN. Metformin is
used as an anti-diabetic drug whose action is to activate the AMPK pathway by
increasing cytosolic AMP through an unknown mechanism (Zhang et al. 2007). HCN is
positively modulated by cAMP in a phosphorylation-independent manner (Robinson and
Seigelbaum 2003). It is therefore of interest to consider the possibility that metformin’s
mechanism of action may be modulating the pool of cytosolic cAMP available to HCN
channels, thus reducing their activity at rest and upon the hyperpolarization phase of
action potentials.

Phosphodiesterases (PDEs) regulate cellular levels (<1 to 10µm) of cAMP by
controlling the rate of its degradation to AMP (Bender and Beavo 2006). Using mouse
oocytes, Downs et al. (2002) demonstrated that the AMP generated by PDE is of a
sufficient amount to activate the AMPK pathway. Since metformin activates the AMPK
pathway by increasing cytosolic AMP and at the same time reduces axonal performance
in the DCMD it may be that metformin activates a PDE, which increases cytosolic AMP
by degrading the cAMP available for HCN function necessary to support high
performance spike train propagation.

PDEs belonging to the PDE4 family have been shown to be involved in signaling
cascades important in learning/memory and PDE4 inhibition with Rolipram enhances
consolidation and retention of long-term memory (Menniti et al. 2006). More
interestingly, inhibition of PDE4 has been suggested to prevent cerebral ischemia-
induced memory deficits in rats assessed using both Morris water-maze and step-through passive avoidance tests (Li et al. 2011). It would be interesting to see if the PDE inhibitor Rolipram would counteract the effect of metformin on DCMD axon or whether it would help to rescue axonal performance in hypoxic nerve cords. One way to test the hypothesis that metformin exerts its affect through the activation of PDE would be to repeat the metformin experiments performed here, in the presence of the PDE inhibitor Rolipram.

*Conclusion: The virtues of DCMD as a model system*

Understanding how an animal’s nervous tissue and its subsequent behavior changes adaptively to cope with periods of hypoxia is not only of interest to the neuroethologist. Insights gained from the study of how animals adapt to stress has been exceedingly informative to the field of medicine and will more than likely continue to do so *ad infinitum*. Research towards the understanding of how nervous systems function requires not only an understanding of how they operate under ideal conditions but how they respond adaptively to transient metabolic, thermal and mechanical stresses. In the pursuit of this knowledge many animal model systems have been employed and depending on the question one is attempting to answer the model system chosen often means the difference between making progress or not.

Studying the dynamic functioning of axons in mammals is often limited by their small sizes and a general inaccessibility of axons to electrophysiological recording
techniques. The DCMD of locust *L. migratoria* offers a great opportunity for the study of axon physiology owing to its large size and accessibility for both extracellular and intracellular recordings. Previous research into the adaptive response of DCMD to stress has addressed the ability of stress preconditioning to modify the functional limits of DCMD axon (Money *et al.* 2009). Whereas research addressing the role of DCMD in behaviour has established a link between the signals DCMD propagates and escape jump behavior observed in response to looming stimuli (Fotowat and Gabbiani 2011). In this thesis I sought to investigate changes in axonal performance in response to hypoxia.

I report conduction velocities and peak frequencies recorded from normoxic preparations which are nearly double that of previous reports. Given that the values I obtained from hypoxic preparations were nearly identical to those previously reported in studies assessing the network connections of DCMD it is likely that the significance of DCMD inputs has previously been underestimated. I have shown that performance is reduced in response to hypoxic stress, however further studies are required to confirm that reduced performance is the result of reduced permeability, that reduced permeability is indeed protective and adaptive. Furthermore it will be important to determine how changes in permeability are mediated and which conductances are involved.

I have presented evidence indicating a possible role for the HCN channel and/or the AMPK pathway’s involvement in mediating changes in performance and speculated on the possible role of PDEs in mediating axonal performance. Clearly further investigations are needed however the answers to such investigations will likely go a far way in terms of furthering our overall understanding of axon physiology.
LITERATURE CITED


