



University of
Nottingham

UK | CHINA | MALAYSIA

School of Life Science

Masters of Research in Neuroscience

2016-2017

Thesis Title:

**Schwann Cell-Axon Metabolic Interactions
of the Mouse Sciatic Nerve**

Author:

Miss Laura Rich

Word count: 25,958

Acknowledgement

I would like to express my gratitude to my supervisor Dr Angus Brown for allowing me this opportunity and the constant support he has provided throughout including, but by no means not limited to, the routine “tea time” breaks! I would also like to express my thanks for the many experiences, in addition to lab work, having Angus as a supervisor has given me; most importantly introducing me to Dr Bruce Ransom- something I will always be proud of. I look forward to publishing my first paper with this data and starting my PhD alongside Angus.

Furthermore I would like to acknowledge Dr Maxine Fowler for her expertise in immunohistochemistry and Western blotting, and willingness to help, without which the final piece of the puzzle could not have been done.

Finally, thank you to my parents for all their encouragement and enthusiasm, without whom I would not have been able to undertake this Research Masters, and to Helena who has always been there and happy to pick me up after late nights in the lab.

Table of Contents

Abstract	5
Chapter 1: Introduction	6-45
1.1. Brain energy metabolism	6
1.1.1. <i>Uptake of energy substrates into the brain</i>	6
1.2. Glucose metabolism in the central nervous system	8
1.2.1. <i>Contribution of astrocytes to central nervous system energy metabolism</i>	11
1.3. Other substrates used for central nervous system energy metabolism	14
1.3.1. <i>Glycogen</i>	14
1.3.2. <i>Lactate</i>	19
1.3.2.1. <i>The signal to stimulate lactate transfer from astrocytes to neurones</i>	22
1.3.3. <i>Fructose</i>	26
1.4. Peripheral nervous system energy metabolism	32
1.4.1. <i>The sciatic nerve</i>	32
1.4.2. <i>Glucose metabolism in peripheral nerves</i>	36
1.4.3. <i>Glycogen metabolism in peripheral nerves</i>	37
1.4.4. <i>Lactate metabolism in peripheral nerves</i>	39
1.4.5. <i>Fructose metabolism in peripheral nerves</i>	42
1.5. Aim and hypotheses	44

Chapter 2: Methodology	46-61
2.1. Sciatic nerve	46
2.2. Electrophysiology	47
2.3. Lactate biosensors	51
2.4. Data analysis	54
2.5. Western Blotting	56
2.6. Immunohistochemistry	59
2.7. Experiment timeline	61
 Chapter 3: Results	 62-79
3.1. The effect of withdrawal of an exogenous energy substrate on A and C fibre conduction	62
3.2. The effect of metabolic pathway inhibitors on A and C fibre conduction	64
3.3. The use of fructose as an energy substrate to maintain A and C fibre conduction	66
3.4. The use of fructose as an energy substrate to recover A and C fibre conduction	69
3.5. The effect of 4-CIN on the use of fructose as an energy substrate to maintain and recover A and C conduction	71
3.6. Lactate release from the sciatic nerve in the presence of glucose and fructose	75
3.7. The expression of fructokinase in the sciatic nerve	78

Chapter 4: Discussion	80-98
4.1. Schwann cell glycogen-derived lactate supports A fibre, but not C fibre, conduction during removal of an exogenous energy substrate	81
4.2. Differential requirement for glycolytic and oxidative metabolism between fibre types and Schwann cells	83
4.3. A fibres require a higher concentration of fructose than C fibres	87
4.4. Possible role of Schwann cell fructose-derived lactate in supporting A fibre conduction	90
4.5. Fructokinase expression in the sciatic nerve	94
4.6. Future directions	96
4.7. Limitations	98
 Chapter 5: Conclusions	 99
 Abbreviations	 100
 References	 101-106

Abstract

In the Peripheral nervous system (PNS), akin to the central, glial cells, Schwann cells and astrocytes, respectively, contain glycogen, which is glycolytically metabolised to lactate and transported to axons for oxidative metabolism. In the PNS, however, only the myelinated A fibres, not the unmyelinated C fibres, benefit. Using *in vitro* electrophysiology to simultaneously record A and C fibre conduction of the mouse sciatic nerve, I investigated the metabolic interactions between Schwann cells and axons, particularly during fructose metabolism.

Increased maintenance of A fibre conduction compared to C fibres in the absence of energy substrate confirmed that A fibres benefit from glycogen-derived lactate. Metabolic inhibitors, iodoacetate and cyanide, implied a preference for both glycolytic and oxidative metabolism by the A fibres, whereas C fibres survive solely on oxidative metabolism, and Schwann cells on glycolysis.

C fibres required lower fructose concentrations (10mM) than the A fibres, but even in the presence of higher concentrations (20mM) recovery of A fibre conduction as a result of an absence of energy substrate was slow. This implied C fibres would express fructokinase (low K_m) not the A fibres, but instead would metabolise fructose via hexokinase (high K_m). However, immunohistochemistry revealed fructokinase expression by all axons and not Schwann cells. Inhibition of lactate transport with fructose as the sole energy substrate reduced the maintenance and recovery of A, but not C, fibre conduction, suggesting a need for fructose-derived lactate from the Schwann cells by A fibres. Despite this, lactate biosensors recorded lactate released from the nerve into the surrounding bath prior to recovery of A fibre conduction with 20mM fructose. A possible explanation for the slow recovery and presence of extracellular lactate is the apparent glycolytic requirement of the A fibres, which is delayed due to the Schwann cells requirement for high concentrations limiting the access to fructose. The results are consistent with a model in which A fibres metabolise fructose directly, as do C fibres, but also metabolise Schwann cell fructose-derived lactate.

Chapter 1: Introduction

1.1. Brain energy metabolism

Energy metabolism is an important physiological process and when carried out by neural cells it contributes to the complex but well-tuned nature of the brain. Firstly, the brain is an energetic organ utilising 15-20% of the cardiac output whilst only contributing to 2% of the whole body weight (Williams & Leggett 1989). In addition neurones exhibit rapid changes in activity levels (Barros et al 2013) and subsequent energy demands thus require an oxygen and energy substrate supply that can instantaneously meet these demands. Finally, brain energy metabolism involves many metabolic pathways and coordinated signalling between cell types to indicate energy demand (discussed throughout). Altogether, this indicates the importance of a constant substrate and oxygen supply to the brain in order to prevent the onset of a coma that can occur within minutes when such a supply is disrupted (Rossen et al 1943).

1.1.1. Uptake of energy substrates into the brain

The brain is supplied with energy substrates, primarily glucose, by the blood. However, a major barrier between the blood and the brain extracellular fluid (ECF) is the selectively permeable blood brain barrier (BBB) (Bear et al 2007). The ability of substrates to cross the BBB depends on their lipophilicity (Squire et al 2013), therefore substrates such as glucose are unable to freely diffuse across the barrier due to their hydrophilic nature, but instead are transported via specific facilitative transporters expressed by endothelial cells that partially - comprise the BBB (Amaral 2013). Two families of metabolic substrate transporters of particular interest are the glucose transporters (GLUTs) and the monocarboxylate transporters (MCTs). These two families consist of many isoforms (55kDa GLUT1 (Vannucci et al 1997) and MCT1 (Halestrap & Price 1999) are the predominant isoforms expressed by the BBB) which transport their respective substrates in a bidirectional manner and are sensitive to concentration gradients not membrane potential (Barros & Deitmer 2010). Moreover, GLUTs are uniporters predominantly for glucose, but can transport

others e.g. fructose, mannose and galactose (Gould & Holman 1993), while MCTs are symporters in that a monocarboxylate is co-transported with a proton (H^+) i.e. they are electroneutral, thus they are affected by, and sensitive to transmembrane pH gradients (Broer et al 1998). The main monocarboxylate transported via MCTs is lactate but, they can also transport pyruvate and ketone bodies such as β -hydroxybutyrate (Broer et al 1998).

The BBB transporter expression varies throughout development. During the pre-weaning period the expression of GLUTs and MCTs are both high but as the brain matures GLUTs become the dominant metabolic transporter expressed by the BBB (Vannucci & Simpson 2003) i.e. glucose is the primary fuel used by the mature brain.

1.2. Glucose metabolism in the central nervous system

Glucose is a six carbon compound and its metabolism is broadly divided into the initial oxygen-independent glycolytic pathway and the latter oxygen-dependent tricarboxylic acid cycle (TCA cycle) and electron transport chain (ETC) (Figure 1.2). Glycolysis occurs in the cytoplasm of the cell and produces two molecules of pyruvate, two ATP and two NADH per glucose molecule under aerobic (sufficient oxygen) conditions, whilst under anaerobic (limited oxygen) conditions the resultant pyruvate is subsequently converted to lactate via lactate dehydrogenase (LDH, requires the reduction of two molecules of NADH to NAD⁺) altogether yielding two molecules of lactate and two ATP per glucose (Champe et al 2008). Under aerobic conditions pyruvate is then oxidatively metabolised in the mitochondrial matrix through the TCA cycle, resulting in three NADH and one FADH₂ per pyruvate (Champe et al 2008). Electrons from the reduction of NADH and FADH₂, formed during glycolysis and the TCA cycle, are then passed along the ETC located in the inner mitochondrial membrane to O₂ (Champe et al 2008). This enables the complexes of the ETC to pump H⁺ from the mitochondrial matrix into the intermembrane space, resulting in a H⁺ gradient which ATP synthase then exploits to form ATP from ADP phosphorylation (Champe et al 2008). Not only can glucose be oxidatively metabolised but it can also, under certain conditions (in selective cell types) be metabolised along the pentose phosphate pathway or stored as glycogen (glycogenesis), the energy store of the brain (Belanger et al 2011).

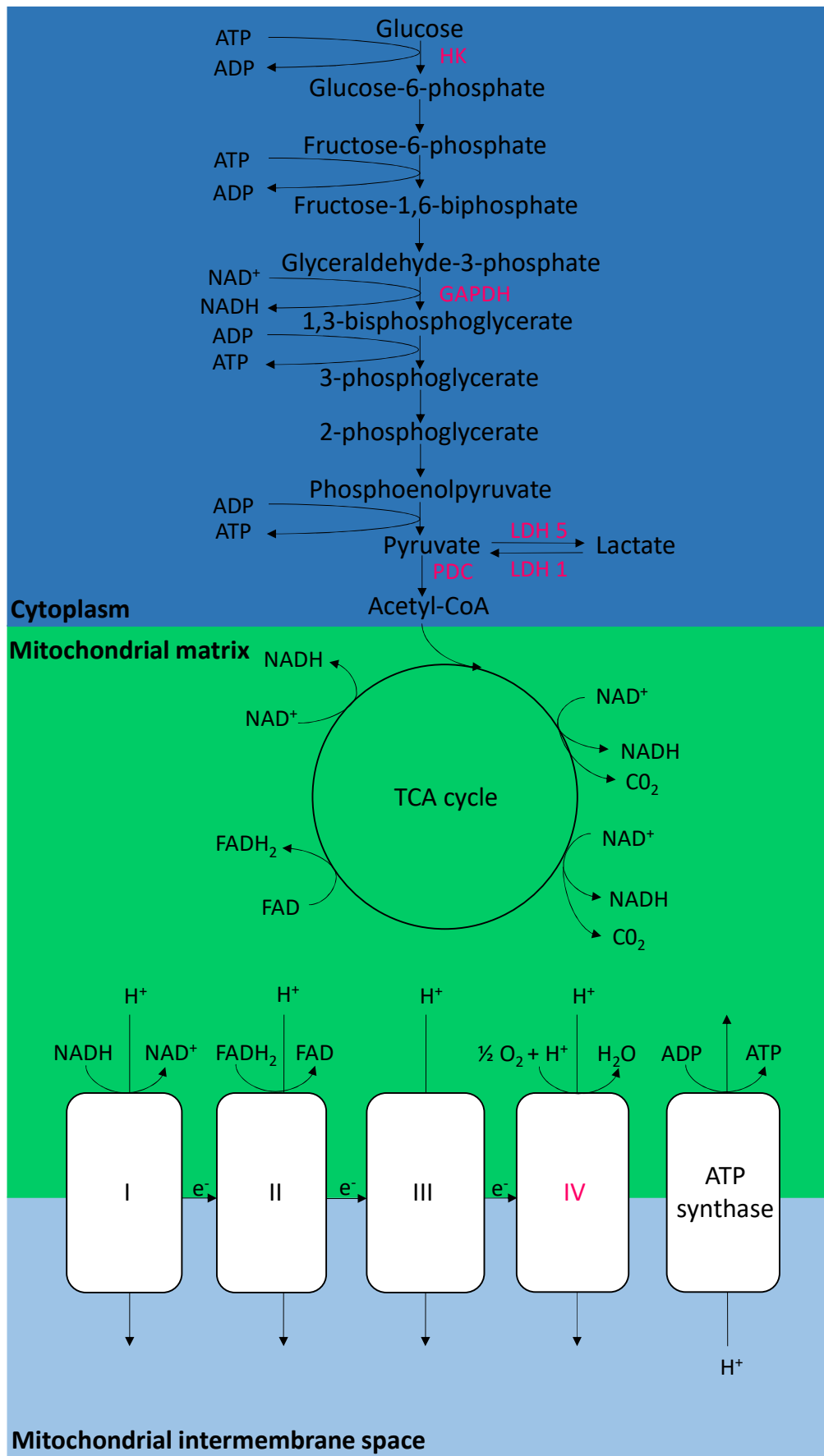


Figure 1.2: Glucose metabolism. Glycolysis takes place in the cytoplasm of the cell and involves both energy requiring (before fructose-1,6-biphosphate) and energy producing phases (after fructose-1,6-biphosphate). After fructose-1,6-biphosphate two of each compound is produced overall resulting in two molecules of pyruvate, two ATP and two NADH. Pyruvate is either converted to lactate or acetyl CoA where it enters the TCA cycle in the mitochondrial matrix. H^+ from the reduction of NADH and FADH are pumped into the intermembrane space by the four complexes of the ETC producing a H^+ gradient used by ATP synthase in order to phosphorylate ADP to ATP. Key sites of action mentioned throughout are highlighted in pink. HK= hexokinase, GAPDH= Glyceraldehyde 3-phosphate dehydrogenase, LDH= lactate dehydrogenase and PDC= pyruvate dehydrogenase complex.

The normal concentration range of glucose present in the blood is between 3.9 and 7.1mM whereas the concentration of brain glucose in the interstitial fluid is between 0.5mM and 1.5mM under normoglycemic conditions (Dienel & Cruz 2004). Hypoglycemia (low blood glucose) is characterised by a systemic blood glucose concentration lower than 2mM, which results in almost 0mM brain glucose concentration, known as cerebral hypoglycemia, due to the rate of consumption overtaking that of transportation (Choi et al 2001) i.e. demand exceeds supply.

Glucose is considered as the primary energy substrate used by the brain, and due to neurones requiring ~94% of the brains' energy to maintain and restore transmembrane ion gradients disrupted during signalling, (Attwell & Laughlin 2001) and the fact that oxidative metabolism produces far more ATP per glucose molecule than glycolysis alone, it is widely accepted that neuronal energy metabolism is a predominantly oxidative event (Belanger et al 2011). However, using positron emission tomography (PET) the ratio of oxygen consumption to glucose utilisation in the brain has been measured at around 5.5, under unstimulated conditions (Hatazawa et al 1988). A value of 6 would imply complete oxidation of glucose to CO_2 and H_2O , thus 5.5 indicates that some glucose is incompletely oxidised to lactate (Dienel 2009), further suggesting that if neurones are exclusively oxidative, then another cell type is at least partially glycolytic (Belanger et al 2011).

1.2.1. Contribution of astrocytes to central nervous system energy metabolism

Since their discovery in 1856 (Virchow) astrocytes were considered to play a simple passive role of structural support within the central nervous system (CNS), resulting in their early name of neuroglia where glial means “glue” (Bouzier-Sore et al 2002). However, during the last two decades they have been ascribed many additional roles and it is now generally accepted that astrocytes perform many dynamic functions and are essential to the functioning of the CNS including brain energy metabolism.

Neurones are prone to many fundamental problems associated with their energy metabolism including: a) their lack of direct contact with cerebral vasculature; the source of glucose and oxygen b) the substantial diffusion distance between their cell body and axons/dendrites c) their deficiency in a stored form of energy substrate; glycogen is toxic to neurones, and d) their activity dependent variations in energy demand (Barros 2013). Although both neurones and astrocytes express glucose transporters, insulin-insensitive GLUT3 and 45kDa GLUT1 respectively, enabling them to take up their own supply of glucose (Vannucci et al 1997), it was Golgi in 1886 who first identified that neuroglia (later to be named astrocytes) in the brain are positioned between neurones and blood vessels, suggesting a nutritive role in which they supply the neurones with energy substrates such as glucose from the circulation (Bouzier-Sore & Pellerin 2013). Moreover, despite the absence of glycogen in neurones it has been found to be predominately located in astrocytic endfeet (Cataldo & Broadwell 1986) in addition to the enzyme glycogen phosphorylase (both the brain and muscle isozyme), which is required for glycogen breakdown (Pfeiffer-Guglielmi et al 2003), implying that they not only possess a store of glucose but are also able to metabolise it. This clearly suggests that glia are ideally positioned and equipped to metabolically support neurones.

Tsacopoulos' et al (1994) work on the honeybee drone retina demonstrated substrate transfer from glial cells to neurones. The honeybee retina was used as a model due to it consisting of one type of neurone; the photoreceptor and one type of glial cell; the outer pigment cell, which is equivalent to the mammalian

astrocyte (Tsacopoulos et al 1994). Glycogen is absent from the photoreceptors, but present in the glial cells, while the density of mitochondria is far greater in the photoreceptors than the glial cells (Tsacopoulos et al 1994). Tsacopoulos et al (1994) showed that glucose-6-phosphate in the glial cells (from glucose phosphorylation and/or glycogen breakdown) is glycolytically metabolised to pyruvate and transaminated by the enzyme ALAT to alanine. Alanine is then released by the glial cells and taken up by the photoreceptors, where ALAT catalyses the reversible transamination reaction back to pyruvate, which can then be oxidatively metabolised in the mitochondria (Tsacopoulos et al 1994). Not only did Tsacopoulos et al (1994) provide support for the theory of metabolic cell-to-cell signalling between glial cells and neurones (Figure 1.2.1), but also demonstrated the presence of metabolic compartmentalisation between the two cell types, in that glial cells display a tendency towards glycolytic metabolism while neurones show a preference for oxidative metabolism (Belanger et al 2011).

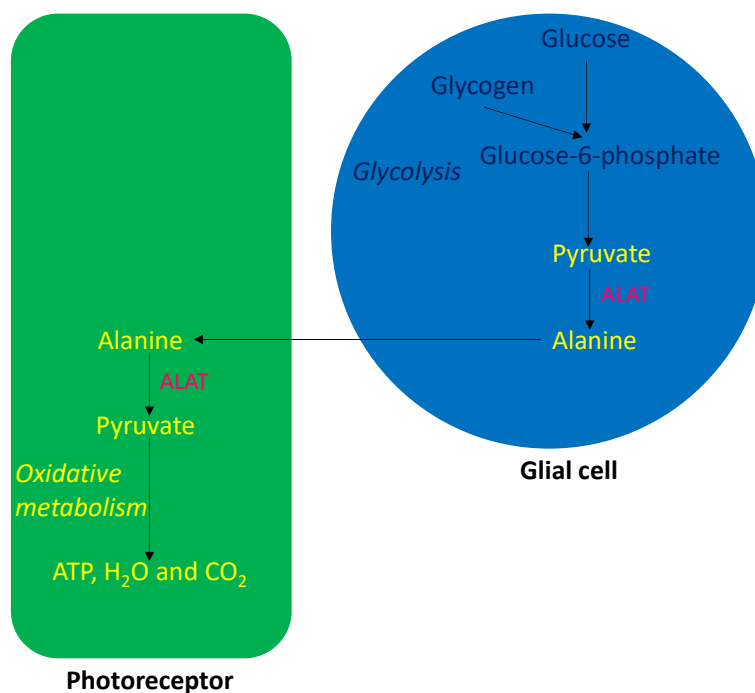


Figure 1.2.1: The glial cell-photoreceptor metabolic interaction in the honey bee drone retina. The glial cell provides the photoreceptor with alanine, the end product of glycolysis, which the photoreceptor oxidatively metabolises.

Investigations into the differential expression of genes associated with energy metabolism have provided further understanding of the cellular metabolic compartmentalisation apparent between astrocytes and neurones. These include a) PFKFB3, a gene that encodes a glycolytic enzyme expressed at a higher level by astrocytes than neurones (Herrero-Mendez et al 2009), b) lactate dehydrogenase (LDH), astrocytes express LDH5, which favourably transforms pyruvate to lactate while neurones and astrocytes express LDH1, which tends to oxidise lactate to pyruvate (Bittar et al 1996), c) the pyruvate dehydrogenase complex (PDC), a group of enzymes that convert pyruvate to acetyl-CoA for entry into the TCA cycle tends to be in its inactive (phosphorylated) form in astrocytes while it tends to be present in its active (dephosphorylated) state in neurones (Halim et al 2010), and d) the aspartate glutamate complex of the malate-aspartate shuttle in mitochondria, expressed at a lower level by astrocytes than neurones (Ramos et al 2003). Moreover, not only do astrocytes display a preference for glycolysis but the resultant end product tends to be lactate rather than pyruvate even in the presence of sufficient levels of oxygen i.e. aerobic glycolysis; known as the Warburg effect (Bouzier-Sore & Pellerin 2013, Magistretti & Allaman 2015).

1.3. Other substrates used for central nervous system energy metabolism

Glucose is widely accepted to be the principal energy substrate of the brain, however it is now also thought that other substrates can be used by the brain under certain conditions (Belanger et al 2011). There has been particular emphasis on the use of lactate by neurones, due to its release by astrocytes as an end product of their own energy metabolism. Moreover research has revealed that astrocytes provide neurones with a source of energy during times of insufficient substrate supply through glycogen breakdown to lactate. Finally, investigations into fructose as a sufficient energy substrate for CNS tissue are beginning to emerge.

1.3.1. Glycogen

Glycogen is comprised of many glucose residues, the majority of which are joined by α -1,4-glycosidic bonds, with about every 10th residue joined by an α -1,6-glycosidic bond, forming a very large, branched polymer (Champe et al 2008). Glycogen synthesis (glycogenesis) is carried out by the enzyme glycogen synthase (α -1,4 linkages) while the key enzyme involved in glycogen breakdown (glycogenolysis) is glycogen phosphorylase (Champe et al 2008). Glycogen phosphorylase cleaves glycogen at α -1,4-glycosidic bonds releasing glucose-1-phosphate, which is then converted to glucose-6-phosphate before entering glycolysis (Figure 1.3.1.i) (Champe et al 2008).

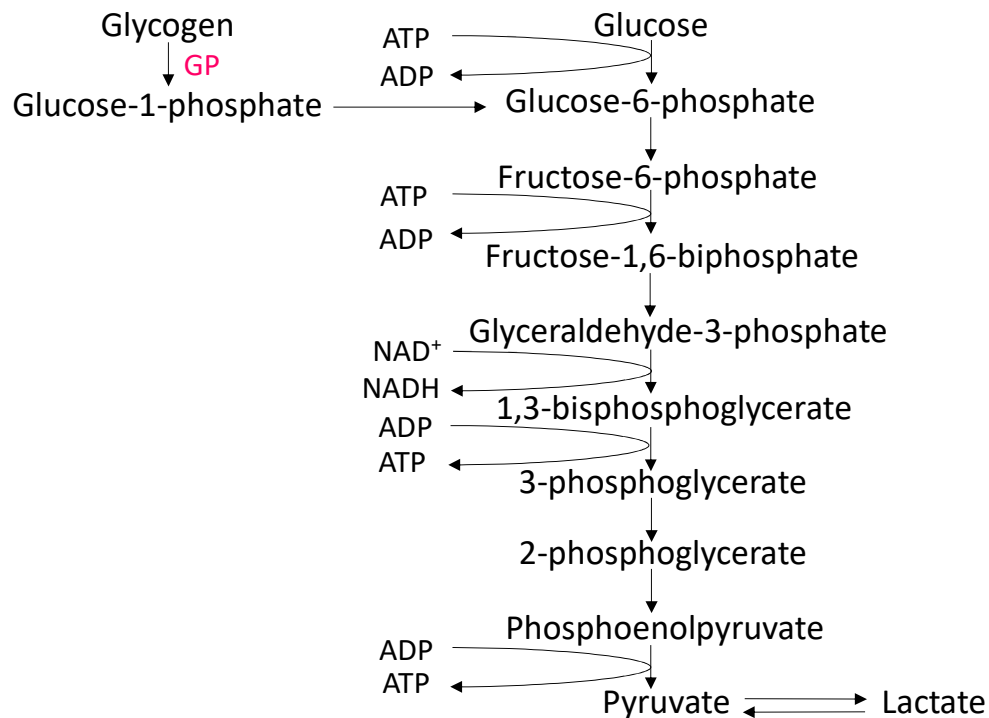


Figure 1.3.1.i: Glycogen breakdown. Glycogen is broken down to glucose-1-phosphate by glycogen phosphorylase (GP) then converted to glucose-6-phosphate which then enters the glycolytic pathway.

Glycogen in the brain, measured using ^{13}C NMR spectroscopy, is present at a concentration of around 2 and $3\mu\text{mol/g}$ tissue (Choi et al 2003), which is significantly lower than its concentration in the muscle ($30\text{--}100\mu\text{mol/g}$ tissue) and liver ($100\text{--}500\mu\text{mol/g}$ tissue) (Shulman et al 1995), hence the initial neglect of research into brain glycogen. However, due to experimental procedures, in particular the tissue extraction method, the typically reported concentration of brain glycogen may be an underestimation of the true value ($8\text{--}12\mu\text{mol/g}$) (Cruz & Dienel 2002). Despite the expression of glycogen synthase by neurones (Pellegrini et al 1996), it is the astrocytes that house the glycogen in the brain (Cataldo & Broadwell 1986) and possess the ability to both synthesise (Pellegrini et al 1996) and metabolise it (Pfeiffer-Guglielmi et al 2003).

Initial investigations into the role of brain glycogen involved insulin-induced hypoglycaemia. In dogs it was found that excess insulin resulted in a decline in the glycogen concentration of brain areas with the highest metabolic rate first (Chesler & Himwich 1944). Moreover, *in vivo* NMR spectroscopy studies showed

that during hypoglycaemia only once the brain glucose levels had dropped to zero did the brain glycogen content start to fall (Choi et al 2003). However intriguing, these studies only show an indirect relationship between brain glycogen and function, rather than a functional relationship. Studies using cell cultures provided a glimpse of a functional relationship from the finding that the increased survival time of neurones co-cultured with glial cells compared to those cultured in the absence of glial cells (Whatley et al 1981) was a consequence of the glycogen present in the astrocytes (Swanson & Choi 1993). This was evident from the prevention of neuronal cell death when the co-localised cell culture was preincubated in insulin (increases glial glycogen levels) but not noradrenaline (reduces glial glycogen content) before glucose deprivation (Swanson & Choi 1993). However, cell culture studies should be treated with caution (due to the absence of cell-to-cell signalling with other cell types that may occur *in vivo*) thus subsequent studies have built upon these first culture studies using *ex vivo* preparations such as the optic nerve.

The optic nerve is a central nervous system white matter tract that has been extensively used, in particular by the Waxman and Ransom research groups, to study, among other things, the ability of astrocytic glycogen to support axon conduction. It is an ideal model not only because of its accessibility, but also because white matter structures are far simpler than grey matter structures due to being devoid of cell bodies and glutamatergic synapses. The optic nerve preparation consists of axons, oligodendrocytes and astrocytes but electron microscopy images revealed the presence of glycogen only in astrocytes, not axons or oligodendrocytes (Wender et al 2000), thus glial cell-axonal metabolic interactions in the CNS have focused on astrocytes.

As glycogen is a storage compound, its role in the CNS was first investigated under pathological conditions i.e. aglycemia. A functional relationship between brain glycogen and axon conduction was determined through optic nerve conduction measured as the area of the compound action potential (CAP) which has a characteristic 3 peak profile (Figure 1.3.1.ii). Initial studies using rat optic nerve (RON) found that simply subjecting it to aglycemia resulted in

maintenance of the CAP for about 30 minutes before it began to decline and fall to zero by 60 minutes, a pattern that corresponded with astrocytic glycogen content which also fell to its nadir after approximately 30 minutes of aglycemia (Wender et al 2000). Through manipulation of the concentration of exogenously applied glucose, it was also determined that glycogen content was linearly related to glucose concentration and that the concentration of astrocytic glycogen determined the latency to CAP failure during aglycemia i.e. increased glycogen increased latency to failure (Wender et al 2000). This clearly implies that glycogen supports axon conduction in the absence of glucose.

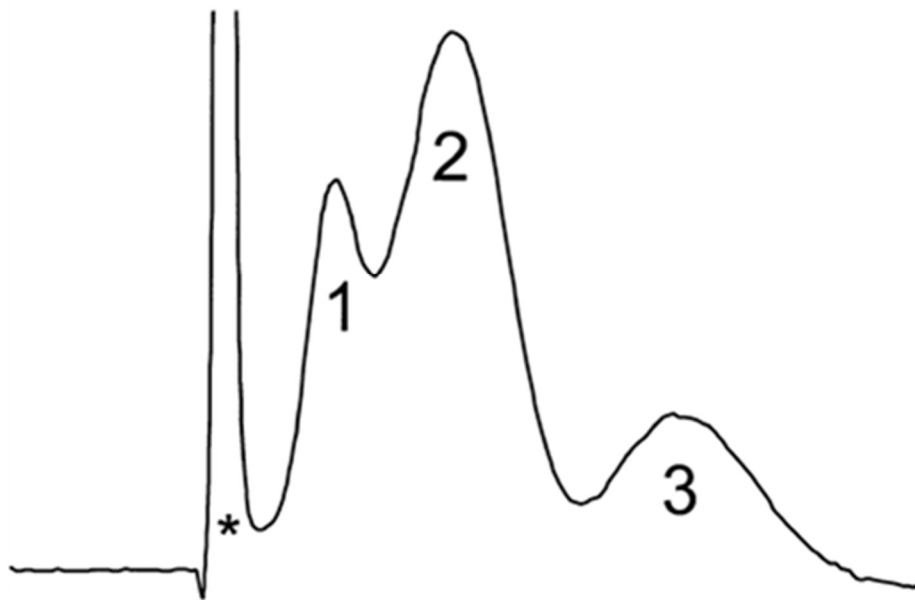


Figure 1.3.1.ii: The 3 peak (1, 2 and 3) optic nerve CAP occurs immediately after the stimulus artefact (*) (Modified from Allen et al 2006).

Optic nerve studies now use mouse optic nerve (MON) in preference to the rat for the following reasons: a) it is smaller thus diffusion is more efficient, b) the tendency for transgenic models to be mice, and c) when the experiments performed on the RON were repeated on the MON quantitatively similar results were obtained; 30 vs 20 minutes latency to failure during aglycemia (Brown et al 2003). Subsequent MON studies involved a glycogen phosphorylase inhibitor; isofagomine (Brown et al 2005). Isofagomine had no effect on the CAP during

normoglycemic (10mM glucose) conditions, but its presence during aglycemia attenuated the latency to MON CAP failure (~11 minutes) compared to aglycemia alone (~20 minutes), even when the glycogen content was increased (Brown et al 2005). These results further confirm the importance of glycogen breakdown in sustaining axon conduction during aglycemia.

Brown et al (2003) also showed that glycogen is required to sustain axon conduction when glucose is available but only at low concentrations (hypoglycemia). Lowering the concentration of glucose supplied to the MON from 10mM to 2mM resulted in the maintenance of the CAP, but when glycogen was depleted prior to perfusion of 2mM glucose the CAP failed rapidly (Brown et al 2003).

The research described so far only focused on the role of glycogen in supporting axon conduction under pathological conditions implying glycogen is present as an emergency energy store, but due to the relatively uncommon occurrence of systemic hypoglycemia it could be argued that this is not the extent of its function (Brown et al 2004). A role for brain glycogen during physiological conditions is still debated, but there is evidence suggesting its importance including; its synthesis during sleep and subsequent breakdown on waking (Benington & Heller 1995), its depletion as a result of sleep deprivation (Franken et al 2003), its increase under anaesthetic (Rosengarten 1970) and its role in memory consolidation (Gibbs et al 2006). Again these findings only imply an indirect relationship rather than a functional relationship.

Using the MON Brown et al (2003) investigated whether astrocytic glycogen provides energy for axons during the physiological condition of increased neuronal activity. This was achieved by subjecting the MON to high frequency stimulation (HFS) (100Hz for 4 minutes) resulting in the maintenance of the CAP during normoglycemic conditions (Brown et al 2003). However, both depleting the glycogen content of the MON prior to (Brown et al 2003), and the addition of isofagomine during (Brown et al 2005) HFS and 10mM glucose, caused the CAP to fall. This implies glycogen may play a role in providing energy during increased neuronal activity when normoglycemic levels of glucose alone cannot meet

these demands i.e. there is a requirement for supplementary glycogen during physiological conditions.

1.3.2. Lactate

The three carbon, electron rich compound lactate is the end product of glycolysis that can no longer be directly metabolised further unless converted back to pyruvate by the enzyme LDH (Champe et al 2008). In addition to being described as a metabolic dead-end it has been a long held view that lactate is a waste/toxic product of energy metabolism (Bergersen 2007), and that the increase in tissue lactate seen during increased neuronal activity is the result of insufficient oxygen supply. However, it is now known this is not the case, but instead the Warburg effect of astrocytes is enhanced by neuronal activity (Bouzier-Sore & Pellerin 2013) and neurones can then use this lactate as an energy substrate (Bergersen 2007). This is further supported by its presence in the brain interstitial fluid at a similar concentration to that of glucose (0.5-1.5mM) (Dienel & Cruz 2004).

A substantial amount of research has been conducted into lactate as an alternative energy substrate for use by neurones in the CNS, with even the earlier studies from the 1950's highlighting its effectiveness. For example, McIlwain (1953) demonstrated that 30mM lactate was able to sustain the high respiratory levels of human cerebral cortex seen with glucose and enable responses to applied electrical stimulation. More recent investigations using MON have also provided functional evidence that lactate is able to support neuronal activity, showing that simply switching the exogenously supplied energy substrate from 10mM glucose to 20mM lactate resulted in both maintenance and complete recovery of the CAP (Brown et al 2003). It is clear that lactate is a sufficient energy substrate for neurones in the CNS but the question still remains about its source. Using cultured astrocytes, spectrometry and NADH (produced by the addition of LDH) as an indicator of lactate in the surrounding media Walz and Mukerji (1988) were the first to show that astrocytes release significant amounts of lactate into the extracellular space (ECS), assigning astrocytes as the source of lactate for neurones.

As already discussed, axons benefit from glycogen stored in astrocytes, particularly during times of energy substrate deprivation, but glycogen itself is an extremely large compound, too big to be passed directly from astrocytes to axons, thus it must first be metabolised to some form of metabolic intermediate such as lactate (Wender et al 2000). Evidence at a molecular level to support this hypothesis comes from the cell type specific expression of MCT isoforms in the brain; neurones tend to express MCT2 (Debernardi et al 2003) while MCT1 (Broer et al 1997, Debernardi et al 2003) and 4 (Rafiki et al 2003) are favourably expressed by astrocytes. The MCT isoforms have different affinities for lactate; MCT1 and 4 have low affinities (K_m of 3.5 and 35mM respectively), while MCT2 has a high affinity for lactate (K_m of 0.7mM) (Bergersen 2007, Broer et al 1999, Broer et al 1998), which can be viewed as determining whether a cell is preferentially oxidative or glycolytic, or whether the cell prefers to export or take up lactate (Bergersen 2007). However due to MCTs being bidirectional the direction of flux depends on the concentration gradients of lactate and H^+ (Bergersen 2007). Moreover, the sodium-bicarbonate cotransporter (NBC), which cotransports two bicarbonates (HCO_3^-) with one Na^+ i.e. generates a transmembrane H^+ gradient, is functionally expressed by astrocytes (Deitmer & Schlue 1989) and has been shown to interact in a cooperative manner with MCT1 to increase lactate transport (Becker et al 2004). Overall, this implies neurones tend to import while astrocytes tend to export lactate.

Wender et al (2000) tested the hypothesis, and provided functional evidence, that astrocytes supply glycogen-derived lactate to axons during pathological conditions such as aglycemia using inhibitors of MCTs. The application of 50 μ M quercetin (blocks lactate release) in the presence of 10mM glucose or 20mM lactate had no effect on the CAP, however during aglycemia it accelerated the latency to failure in comparison to aglycemia alone (Wender et al 2000). On the other hand, 150 μ M α -Cyano-4-hydroxycinnamic acid (4-CIN) or 100 μ M pCMBS (prevent lactate uptake) during normoglycemic conditions had no effect on the CAP, but in combination with 0mM glucose or 20mM lactate resulted in the decline of the CAP (Wender et al 2000). Overall, these results indicate that

astrocytic glycogen is broken down to lactate and transferred to axons to be oxidatively metabolised in order to sustain axon conduction during aglycemia, but that lactate uptake into axons is not essential during normoglycemia (Figure 1.3.2) (Wender et al 2000). The same theory was thought to apply to the pathological condition of hypoglycaemia (Brown et al 2003). Supplying the optic nerve with 150 μ M 4-CIN and 2mM glucose resulted in a rapid fall in the CAP as seen when 2mM glucose is supplied after glycogen depletion, suggesting astrocytic glycogen-derived lactate provides the energy to maintain axon conduction during hypoglycaemia (Figure 1.3.2) (Brown et al 2003).

Again, the above only describes axonal use of glycogen-derived lactate from astrocytes during pathological conditions, however it is known that glycogen is required during increased neuronal activity (Brown et al 2005, Brown et al 2003) suggesting that it is actually glycogen-derived lactate that provides the energy to meet the increased metabolic demand of the axons (Figure 1.3.2). This is supported by the significant fall in the CAP seen during HFS under normoglycemic conditions, in the presence of either 50 μ M quercetin or 150 μ M 4-CIN (Brown et al 2003).

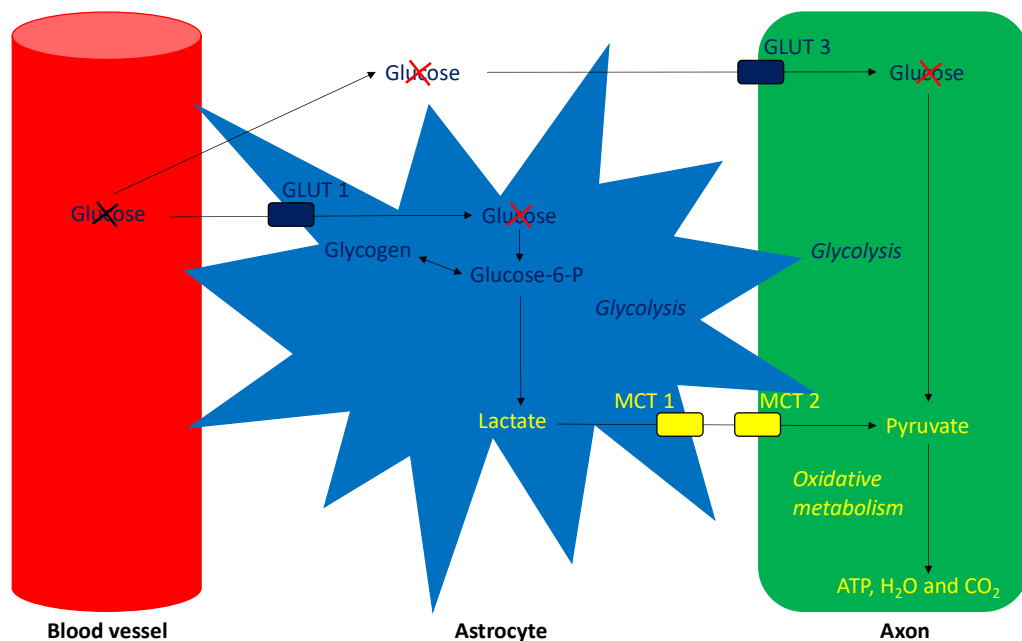


Figure 1.3.2: The metabolic interaction between astrocytes and axons in the central nervous system. Astrocytes provide axons with glycogen-derived lactate particularly during times of absent/low glucose availability (crosses).

1.3.2.1. The signal to stimulate lactate transfer from astrocytes to neurones

Glutamate was first proposed as the signal that stimulates astrocytic glycolysis and subsequent lactate release in order to provide an energy source for neurones, reflected in the astrocyte-neuron lactate shuttle hypothesis (ANLSH) (Figure 1.3.2.1.i) (Pellerin & Magistretti 1994, Pellerin et al 1998). Glutamate is the major excitatory neurotransmitter in the brain, and it is known that astrocytes play a role in clearing glutamate from the synaptic cleft through its cotransport with Na^+ via glutamate transporters and subsequent conversion to glutamine (Pellerin & Magistretti 1994). This results in an increase in intracellular Na^+ which in turn activates the Na^+/K^+ ATPase promoting glucose uptake and astrocytic glycolysis due to its requirement for ATP, thought to be provided by glycolytic enzymes (Pellerin & Magistretti 1994). Lactate as the end product of glycolysis is then released by astrocytes and taken up by neurones for oxidative metabolism (Pellerin & Magistretti 1994).

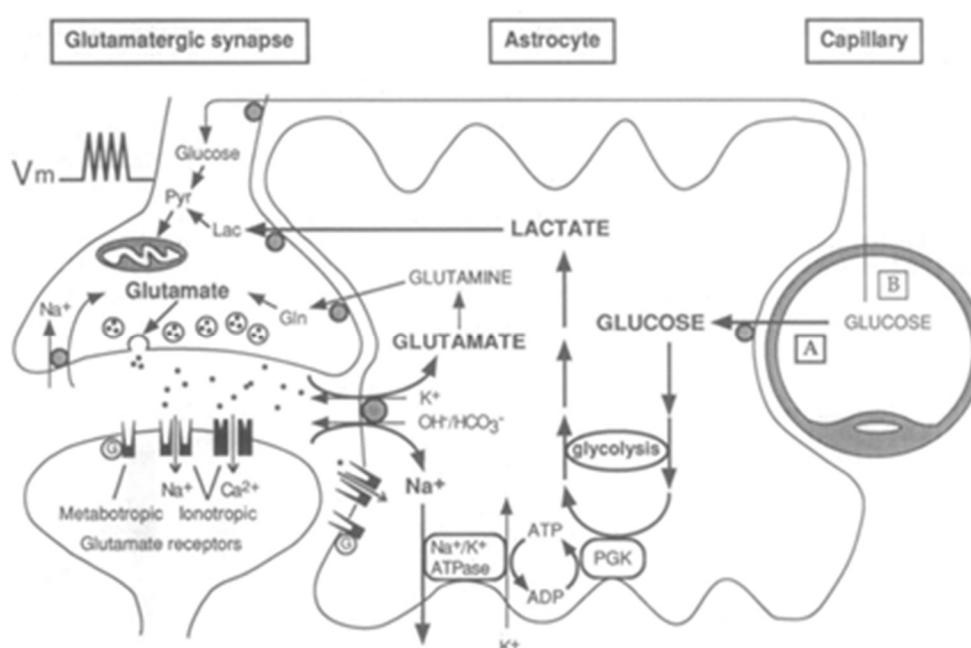


Figure 1.3.2.1.i: The astrocyte-neurone lactate shuttle hypothesis. Glutamate released by neurones is take up by astrocytes along with Na^+ . The rise in intracellular Na^+ then stimulates glucose uptake and glycolysis in order to provide glycolytic ATP for the Na^+/K^+ ATPase. (Modified from Pellerin and Magistretti 1994).

Despite substantial evidence supporting glutamate as the signal coupling glial-neuronal metabolic interactions (Pellerin & Magistretti 1994), it only applies to excitatory glutamatergic neurones and does not incorporate inhibitory GABAergic neurones. Thus Choi et al (2012) went onto investigate K^+ , a ubiquitous cation released from all axons in the central and peripheral nervous system, as the signal that stimulates lactate release from astrocytes using rat hippocampal slices (Figure 1.3.2.1.ii). Soluble adenylyl cyclase (sAC), an enzyme that is more prevalent in astrocytes than neurones and is described as a “metabolic sensor”, when activated creates the second messenger cAMP, which is known to promote astrocytic glycogen breakdown (Choi et al 2012). Using inhibitors and gene KO techniques it was shown that increased extracellular K^+ from baseline 3mM to 10mM causes depolarisation and subsequent entry of HCO_3^- via the NBC transporter into astrocytes (Choi et al 2012). HCO_3^- then activates HCO_3^- sensitive sAC resulting in increased cAMP, glycogen breakdown and subsequent lactate release (Choi et al 2012). This is a universally applicable model because all neurones/axons when activated release K^+ , but glial cells are also selectively permeable to K^+ (Kuffler & Potter 1964) thus are sensitive to changes in K^+ , in addition as described previously the NBC interacts with MCT1 present on astrocytes promoting lactate release.

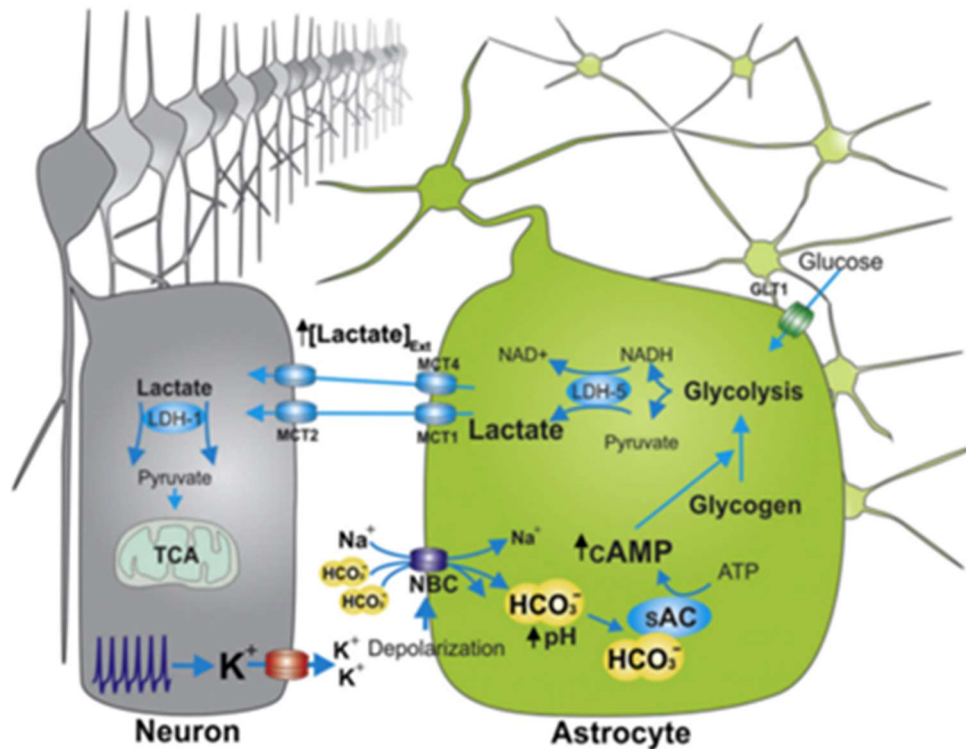


Figure 1.3.2.1.ii: K^+ acts as a metabolic signal between axons and astrocytes. K^+ released from axons, causes HCO_3^- uptake through the NBC into astrocytes activating sAC and triggering glycogen breakdown to lactate via cAMP. (Modified from Choi et al 2012).

A third molecule suggested to act as a metabolic signal between neurones and astrocytes is ammonium (NH_4^+), which is also released by neurones as result of converting glutamine to glutamate (Figure 1.3.2.1.iii) (Lerchundi et al 2015). The study that provided significant evidence towards this theory used a range of genetically encoded FRET nanosensors, which were expressed by astrocytes, enabling intracellular recordings of different energy substrates and pH (Lerchundi et al 2015). Unlike K^+ and glutamate, NH_4^+ was not found to increase astrocytic glycolysis despite resulting in increased extracellular lactate, but instead acted to inhibit pyruvate flux into astrocytic mitochondria (Lerchundi et al 2015). Astrocytes take up NH_4^+ released from neurones where it enters the mitochondria (Lerchundi et al 2015). The presence of NH_4^+ in the mitochondria increases the H^+ concentration on the inside, thus reducing the H^+ gradient required for pyruvate transport into the mitochondria (Lerchundi et al 2015).

Pyruvate then builds up in the cytosol of the astrocytes and is subsequently converted to lactate and released for neuronal use (Lerchundi et al 2015). This theory is interesting as it provides evidence for a different route to increased lactate production and release by astrocytes for neurones, in comparison to glutamate and K^+ . NH_4^+ strengthens the cooperative interactions between glial cells and neurones as it is the astrocytes that provide the neurones with glutamine after clearing of glutamate released by neurones from the synaptic cleft, preventing over excitation. However, again like glutamate it appears to be specific to glutamatergic neurones thus may not be considered as a universal metabolic signalling molecule, in contrast to K^+ .

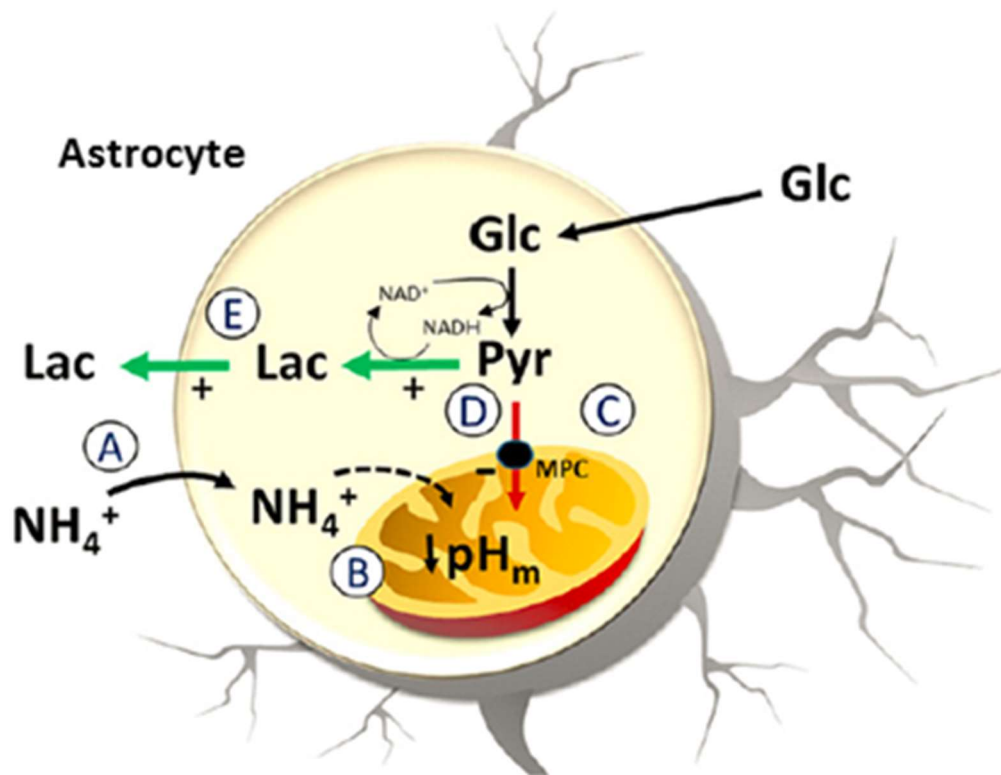


Figure 1.3.2.1.iii: NH_4^+ as the metabolic signal between axons and astrocytes. NH_4^+ released from neurones is taken up astrocytes into the mitochondria. The increased H^+ concentration in the mitochondria prevents pyruvate uptake therefore is converted to lactate in the cytosol and released for axonal use. (Modified from Lerchundi et al 2015).

1.3.3. Fructose

Not only has research been conducted into monocarboxylates as energy substrates (namely lactate) but investigations have also included other monosaccharides, particularly fructose, as alternative energy substrates to glucose used within the nervous system. A natural source of fructose in the diet is fruit, but consumption of fructose has risen since the 1960's due to its presence in many foods in the form of refined sugars such as high-fructose corn syrup (HFCS) (Tappy & Le 2010). HFCS tends to comprise a mixture of both fructose and glucose particularly in the combination of either 42% or 55% fructose (Havel 2005). As a result of this increased intake, fructose metabolism has become a topic of interest particularly due to the apparent relationship with an increased prevalence of obesity and associated diseases including cardiovascular disease and type 2 diabetes (Havel 2005). The metabolism of fructose results in greater lipid synthesis than occurs with glucose metabolism, reduced sensitivity to insulin and reduced production of leptin, all of which contribute to increased energy intake and weight gain (Havel 2005).

Fructose can either be metabolised by fructokinase to fructose-1-phosphate or hexokinase (1st enzyme of the glycolytic pathway) to fructose-6-phosphate, both of which can then enter the glycolytic pathway (Figure 1.3.3.i); fructose-1-phosphate also contributes to the formation of triglycerides (Champe et al 2008). Fructose tends to be metabolised by fructokinase due to its lower K_m i.e. higher affinity for fructose compared to that of the K_m of hexokinase, an exception however is when the concentration of fructose is greater than that of glucose (Champe et al 2008). Fructokinase is also the enzyme abundantly expressed by the liver that metabolises a significant proportion of dietary fructose (Champe et al 2008) resulting in a lower concentration of fructose ($\sim 50\mu\text{M}$) in the blood than glucose (Wahjudi et al 2010). In the liver, 40-50% of fructose is converted to glucose, approximately 25% to lactate, <1% results in triglyceride synthesis (Sun & Empie 2012) and about 20% to glycogen (Tappy & Le 2010).

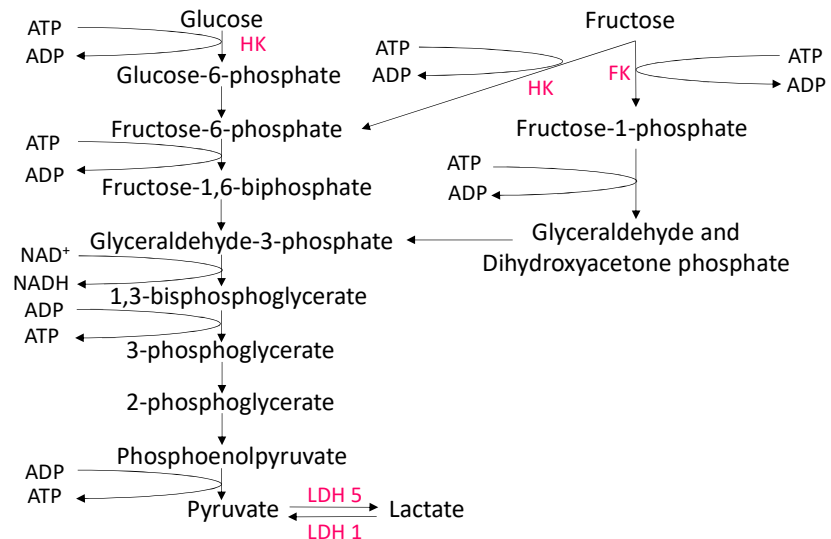


Figure 1.3.3.i: Fructose metabolism. Hexokinase (HK) metabolises fructose to fructose-6-phosphate which then enters glycolysis. Fructokinase (FK) metabolises fructose to fructose-1-phosphate which is further metabolised before entering glycolysis. LDH= lactate dehydrogenase.

Akin to glucose, fructose requires GLUTs to cross cell membranes; however it is the insulin-insensitive GLUT5 isoform that is specialised to transport fructose (Burant et al 1992). In addition to its expression in the BBB (Manty et al 1993), a recent study has reported the presence of GLUT5 on the epithelial cells of the choroid plexus and ependymal cells lining the ventricles in mice, rats and humans, suggesting this is how and where fructose is transported into the brain (Ueno et al 2014). Furthermore it has been shown that fructose can also be made directly in the brain from glucose via the polyol pathway (Figure 1.3.3.ii) (Hassel et al 2015, Hwang et al 2017), suggesting the ability of fructose to accumulate in the brain may not limit its cerebral concentration.

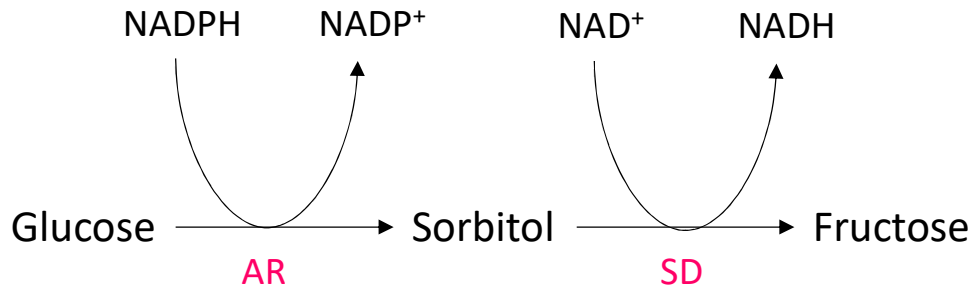


Figure 1.3.3.ii: The polyol pathway. Glucose is reduced to sorbitol via aldose reductase (AR) then converted to fructose by sorbitol dehydrogenase (SD).

The presence of fructose and its transporter suggests fructose can be used as an energy substrate by cells of the nervous system, and is supported by the common finding that 10mM fructose artificial cerebrospinal fluid (aCSF) is able to maintain the high levels of ATP seen during normoglycemic conditions in hippocampal slices (Izumi & Zorumski 2009, Saitoh et al 1994, Wada et al 1998, Yamane et al 2000). This, however, is only indirect evidence suggesting that fructose as an energy substrate is sufficient to support neuronal activity, but in combination with population spike (PS) recording functional evidence is provided. Initially it was found that replacing 10mM glucose aCSF with 10mM fructose resulted in the maintenance of ATP levels but a fall in the PS amplitude to zero within half an hour (Wada et al 1998). However, other longer studies have found that although the PS amplitude did initially drop in the presence of 10mM fructose this spontaneously recovered within 10 to 20 minutes after (almost) complete failure, suggesting fructose is able to maintain neuronal conduction (Saitoh et al 1994, Yamane et al 2000). Yamane et al (2000) concluded that the initial fall in neuronal activity was due to the lower anaerobic metabolic rate of fructose compared to glucose, and the subsequent switch to aerobic metabolism after a sufficient period of hypoglycemia enabled the spontaneous recovery.

Investigations into fructose as a sufficient energy substrate to sustain axon conduction have also been conducted using the MON preparation (Allen et al

2006, Meakin et al 2007). Superfusion of the MON with 10mM fructose resulted in the maintenance of the 2nd and 3rd peaks of the CAP, but decline of the 1st peak after 30 minutes of exposure (Allen et al 2006, Meakin et al 2007). However, supplying the MON with 20mM fructose aCSF enabled maintenance of all 3 peaks, identical to that during normoglycemic (10mM glucose) conditions (Meakin et al 2007). This implies that 10mM fructose is unable to sustain the 1st peak of the MON CAP and based on previous studies (Brown et al 2003) it is likely that it is the astrocytic glycogen, which is broken down to lactate, that is maintaining the 1st peak for the first 30 minutes in 10mM fructose (Meakin et al 2007).

Meakin et al (2007) went on to further investigate the evidence that 10mM fructose is not sufficient to maintain the 1st peak of the MON CAP. Depleting glycogen levels by subjecting the MON to aglycemia caused the CAP to fall after about 20 minutes (Brown et al 2003, Meakin et al 2007). Subsequent perfusion with 20mM fructose aCSF resulted in almost complete recovery of the CAP, again similar to that seen when 10mM glucose is supplied (Meakin et al 2007). On the other hand addition of 10mM fructose after aglycemia resulted in much less recovery of the CAP (~78%) due to the incomplete recovery of the 1st peak, but complete recovery of the 2nd and 3rd (Meakin et al 2007).

Further investigations related to the axon diameters of the MON found that during exposure to 10mM fructose there was a loss of the larger diameter axons (showed signs of pathology as a result of inadequate energy supply), consistent with the loss of the 1st peak (Allen et al 2006). Moreover, immunohistochemical analysis of fructokinase revealed its presence in axons of the MON, but not those of a larger diameter (Meakin et al 2007).

Altogether, it is clear that fructose is only sufficient to maintain complete axon conduction of the MON when present at a higher concentration due to the absence of fructokinase, but presence of hexokinase in the larger diameter axons that contribute to the 1st peak of the CAP.

Research into the ability of fructose to recover a decline in neuronal activity as a result of an absence of energy substrate has further revealed the glial-neuronal interactions underlying the use of fructose as an energy substrate to support neuronal conduction. Izumi and Zorumski (2009) showed that 10mM fructose aCSF was able to efficiently restore (80% recovery) the EPSPs of hippocampal slices with subsequent application of either cytochalasin B (CCB, GLUT inhibitor) or 4-CIN (MCT inhibitor) causing a rapid decline in the EPSPs. This suggested either fructose can be taken up by either transporter directly into the neurones, or that fructose is taken up into glial cells via GLUT where it is glycolytically metabolised to monocarboxylates e.g. pyruvate and lactate, which are transferred to neurones for oxidative metabolism through MCTs (Izumi & Zorumski 2009). The latter hypothesis was confirmed by the fall in EPSPs when 10mM fructose was supplied under anoxic (no oxygen) conditions, which was not restored during reoxygenation (similar to 10mM pyruvate but in contrast to 10mM glucose) (Izumi & Zorumski 2009). The fall and failure of EPSP recovery implies neurones only use fructose as an energy substrate during adequate oxygen supply i.e. oxidative metabolism. Overall, fructose does not appear to be used directly by neurones but indirectly through glial uptake and metabolism to monocarboxylates (Figure 1.3.3.iii). However, CCB does not block the GLUT fructose transporter GLUT5, suggesting this is not the transporter that enables fructose uptake into glial cells in the hippocampus (Izumi & Zorumski 2009). Other possible suggestions include GLUT8 and GLUT11 that are known to transport fructose and are expressed in the brain, however further research is required in order to form a definitive conclusion (Izumi & Zorumski 2009).

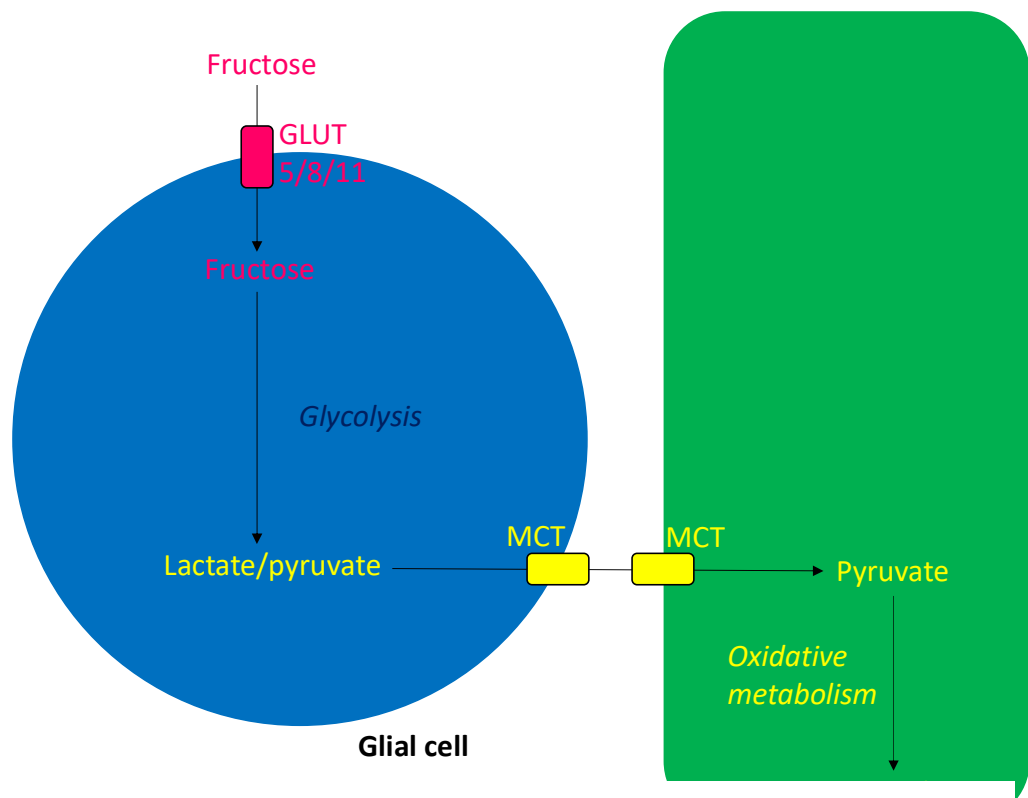


Figure 1.3.3.iii: Glial cell-axon metabolic interactions associated with fructose metabolism. Fructose is taken up by glial cells via GLUT and glycolytically metabolised to a monocarboxylate that is transferred to the axons.

1.4. Peripheral nervous system energy metabolism

As is clear from above, much of the research into the cooperative nature of glial cells and neurones in terms of energy metabolism has been conducted in CNS structures and particularly focused on astrocytes, therefore it is of interest to advance research in the direction of the peripheral nervous system (PNS) in order to determine whether a universal mechanism of axon-glia metabolic interactions exists in both the central and peripheral nervous system. The glial cell of particular interest in the PNS is the Schwann cell, commonly known to myelinate peripheral axons, however a metabolic role is now beginning to emerge. As such the Schwann cell appears to take on the myelinating role of oligodendrocytes and the metabolic role of astrocytes of the CNS.

Despite some older studies identifying that the PNS is capable of using alternative substrates to glucose e.g. lactate (Vega et al 1998) very little research has been conducted into the metabolic nature of the nerves comprising the PNS in terms of the glial-neuronal interactions, thus the recent paper from Brown et al (2012) could be considered as the turning point in this field of research.

1.4.1. The sciatic nerve

Peripheral nerves tend to share a common anatomical structure consisting of bundles of axons (also termed as fibres), some of which are myelinated by Schwann cells (in contrast to the optic nerve, in which all are myelinated in the adult), and blood vessels. The bundles occur due to the presence of 3 different layers of connective tissue; each fibre and its associated Schwann cells are surrounded by endoneurium, fibres are then grouped into fascicles surrounded by perineurium and finally all the fascicles are bundled together by surrounding epineurium to form the nerve (Figure 1.4.1.i) (Low 1976). The vasa nervorum is the microcirculation of peripheral nerves which sends radial branches from the longitudinal extrinsic vessel region to the intrinsic system found in a longitudinal orientation through the epi-, peri- and endoneurium (Figure 1.4.1.ii) (Mizisin & Weerasuriya 2011). Peripheral nerves also have an equivalent BBB called the blood-nerve barrier (BNB) comprised of the endoneurial microvessels within

each nerve fascicle and the associated perineurium (Weerasuriya & Mizisin 2011).

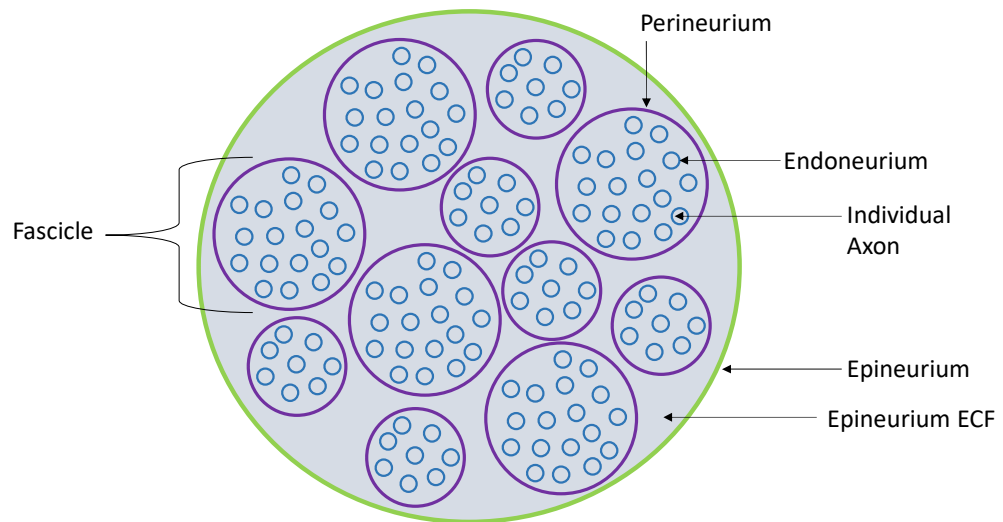


Figure 1.4.1.i: The three layers of connective tissue of a peripheral nerve. The endoneurium surrounds each axon which are bundled together into a fascicle surrounded by the perineurium. All the fascicles are bundled together to form the nerve surrounded by the epineurium. ECF= extracellular fluid.

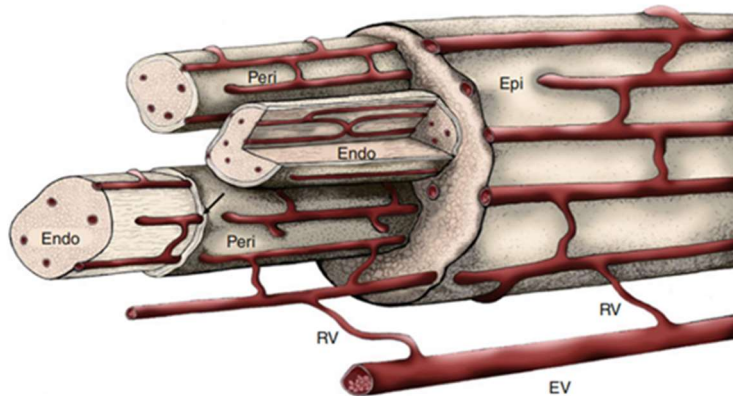


Figure 1.4.1.ii: The vasa nervorum. Radial branches (RV) of the extrinsic vessel region (EV) extend into the epineurium (epi), perineurium (peri) and endoneurium (endo). (Modified from Mizisin & Weerasuriya 2011).

The sciatic nerve, mouse in particular, was chosen as the model to study the metabolic cell-to-cell signalling between glial cells and axons in the PNS because of its ease of access, convenient size for the experimental equipment available, and for comparisons with existing MON and mouse sciatic nerve data. The sciatic nerve is a mixed nerve consisting of motor (6%) and sensory axons (71%) (and 23% sympathetic axons), some of which are myelinated (A fibres) and others that are not (C fibres) (Schmalbruch 1986). The cell in the PNS that myelinates the axons is the glial cell, the Schwann cell, and each Schwann cell myelinates 1 axon, in contrast to oligodendrocytes in the CNS which can myelinate multiple axons (Bear et al 2007). Although C fibres are unmyelinated they are still surrounded by non-myelinating Schwann cells, bundled together to form Remak bundles (Figure 1.4.1.iii) (Kidd et al 2013). The sciatic nerve leaves each side of the spinal cord from the L4-6 region and travels down the leg to the foot (Schmalbruch 1986). Along its path it divides into the tibial and peroneal nerves distal of the trochanter, which then further divide into sural and cutaneous branches (Figure 1.4.1.iii) (Schmalbruch 1986).

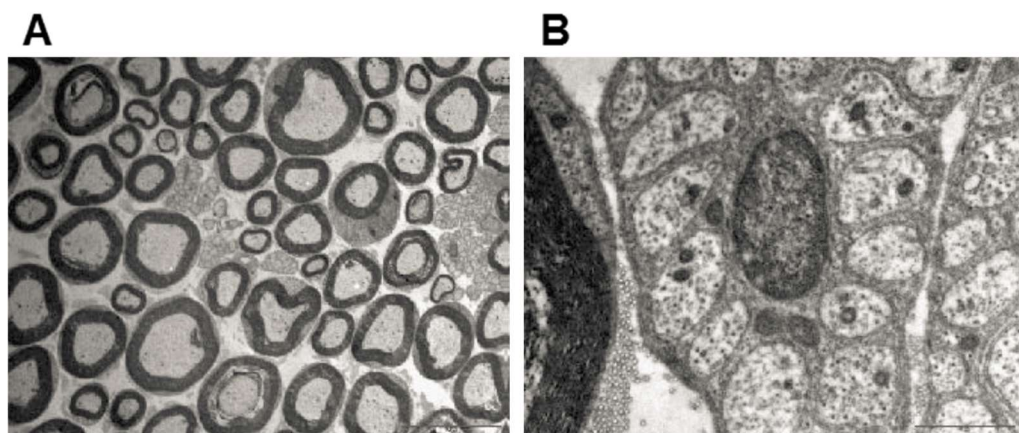


Figure 1.4.1.iii: Electron microscopy of the A and C fibres of the mouse sciatic nerve. **(A)** A fibre axons are myelinated (darker region) by Schwann cells located around the outside. Scale bar= 10 μ m. **(B)** C fibres are encapsulated by Schwann cell cytoplasm into a Remak bundle with the Schwann cell body (darker circle) situated in the middle. Scale bar= 1 μ m. (Dr Angus Brown, personal communication).



Figure 1.4.1.iii: Branching of the sciatic nerve in rat. There are four main branches of the sciatic nerve: tibial (t), peroneal (p), sural (s) and cutaneous (c). (Modified from Schmalbruch 1986).

The sciatic nerve is not only a good model due to its clear anatomical similarities and differences to the CNS optic nerve structure, allowing comparisons, but also because it is particularly susceptible to damage during diabetes; a metabolic disorder. This damage is known as diabetic neuropathy and is thought to be consequence of the high blood glucose levels (NIDDK 2013). There are many types of diabetic neuropathy but peripheral neuropathy is the most common resulting in pain and loss of sensation particularly in the feet and legs i.e. sciatic nerve damage (NIDDK 2013). Thus understanding the metabolic interactions between the Schwann cells and axons under normal, physiological conditions may provide insight into the metabolic cell signalling that is disrupted during metabolic disorders such as diabetes.

1.4.2. Glucose metabolism in peripheral nerves

For any metabolic substrate present in the circulation to be taken up by peripheral nerve axons it has to cross the multi-layered perineurium and the tight junction linked endothelial cells of the endoneurial microvessels of the BNB (Mangia et al 2013). Many studies have shown that GLUT1 is expressed on the perineurium (Magnani et al 1996, Takebe et al 2008) and BNB (Orte et al 1999) indicating transporters are present to transport glucose into the endoneurial compartment (Magnani et al 1996). However, for glucose to then reach the energy demanding nodes of Ranvier of myelinated axons, it must cross the paranodal Schwann cell and axolemma, suggesting the presence of transporters which Magnani et al (1996) investigated in rat sciatic and sural nerves. From broad immunoblot analysis of sciatic nerves GLUT1 was found to be predominately expressed particularly at the 46-53 kDa band, whereas GLUT3 was only found to be significantly expressed by older (11 and 13 month) animals (Magnani et al 1996). Further analysis using immunocytochemistry of sural nerves confirmed the presence of GLUT1 on the perineurium and endoneurial microvessels, and demonstrated its expression on Schwann cell membranes and cytoplasm, particularly at the nodes of Ranvier and Schmidt-Lanterman incisures (Figure 1.4.2) of myelinated axons (Magnani et al 1996). GLUT3 (in 13 month-old rats) was also localised to the perineurium, endoneurial microvessels and paranodal Schwann cells akin to GLUT1, but in contrast GLUT3 was also found to be expressed by axonal membranes, in particular at the nodal axolemma i.e. GLUT1 is expressed by Schwann cells and the BNB, while GLUT3 is expressed by Schwann cells and myelinated axons, implying a parallel phenomenon to CNS GLUT expression, however GLUT3 expression appears to be related to development in the PNS (Magnani et al 1996). Overall, because GLUT1 is not expressed by microvessels of the epineurium (Takebe et al 2008), that also lack a BNB, it can be concluded that glucose passively diffuses from the epineurial microvessels into the ECF of the epineurium (Allt & Lawrenson 2000) where it is transported by GLUT1 into the endoneurial compartment and Schwann cells or via GLUT3 into myelinated axons (Magnani et al 1996).

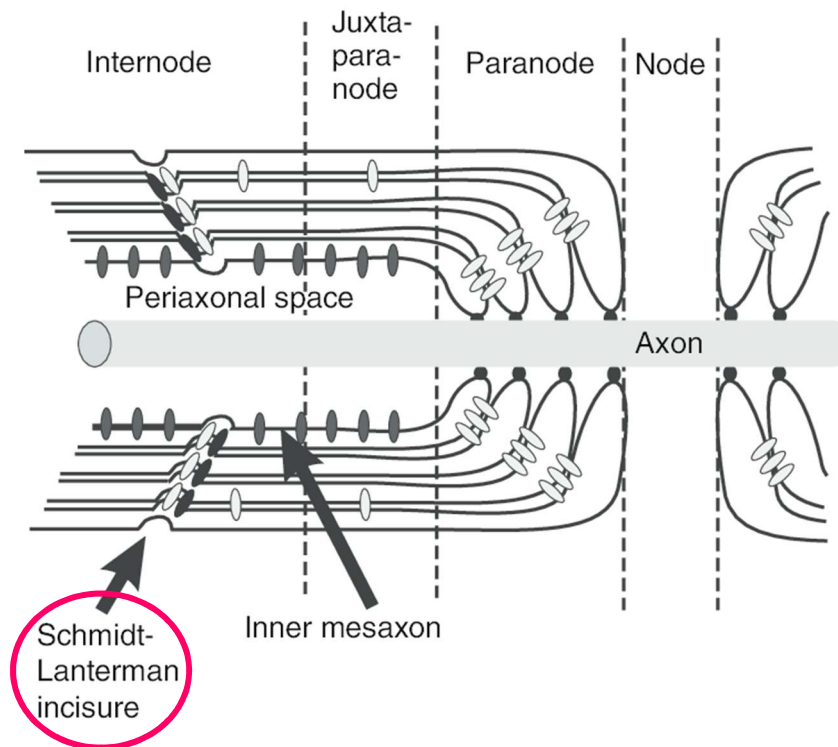


Figure 1.4.2: Schmidt-Lanterman incisure of Schwann cells. Schwann cell cytoplasm that forms non-compact myelin and disrupts compact myelin. GLUT1 and MCT1 (see later) are expressed here. (Modified from Abrams and Rash 2009).

1.4.3. Glycogen metabolism in peripheral nerves

Research has already been conducted into the metabolic interactions between axons and Schwann cells of the mouse sciatic nerve, with a particular focus on glycogen, based on the previous data collected using the MON (Brown et al 2012).

From examination of the mouse sciatic nerve glycogen was found to be present at a concentration of around 8.6 pmoles/ μ g protein under normoglycemic conditions, which interestingly is significantly greater than that of the optic nerve, present at about 6.5 pmoles/ μ g protein (Brown et al 2012). Further analysis revealed the specific localisation of glycogen granules to the Schwann cell cytoplasm rather than the axons i.e. comparable to its presence in the

astrocytes of the CNS, however the majority of Schwann cells that contained glycogen were the myelinating Schwann cells that ensheath the A fibres, rather than the non-myelinating Schwann cells surrounding the C fibres (Brown et al 2012). In addition to glycogen, glycogen phosphorylase was also located to the Schwann cells (Brown et al 2012, Pfeiffer-Guglielmi et al 2007) suggesting that they both house and metabolise glycogen; similar to astrocytes in the CNS. An important point to note here is that glycogen phosphorylase was also found to be expressed by axons despite the absence of glycogen implying the expression of an enzyme alone does not always indicate the presence of its substrate (Brown et al 2012).

Brown et al (2012) went on to determine the functional ability of Schwann cell glycogen to provide energy for axonal activity during aglycemia through recording the CAP responses of the A and C fibres. Simply depriving the sciatic nerve of glucose revealed a significant difference between the latency to failure of the A and C fibres, in that the A fibres continued to function for about 2 hours while the C fibres only lasted about 40 minutes before starting to fail (Brown et al 2012). Electrophysiology recordings in combination with glycogen assays showed the content of glycogen within the sciatic nerve during aglycemia fell progressively to its nadir over 2 hours coinciding with the onset of fall in the CAP of the A fibres, but not the C fibres, implying that it is only the A fibres and not the C fibres that benefit from the Schwann cell glycogen (Brown et al 2012). This phenomenon was further investigated by increasing and decreasing the level of glycogen present at the onset of aglycemia, which was found to prolong and hasten, respectively, the latency to failure of the A fibre CAP, but have very little effect on the C fibre latency to failure (Brown et al 2012). Furthermore the application of 1,4-dideoxy-1,4-imino-d-arabinitol (DAB), a glycogen phosphorylase inhibitor, during aglycemia resulted in the acceleration of the A fibre CAP latency to failure, comparable to that of the C fibres during aglycemia, but had no effect on the C fibre latency to failure (Brown et al 2012). Overall it appears that the glial-axonal metabolic interactions are similar between

astrocytes and axons in the CNS, and Schwann cells and A fibres in the PNS, but not to the non-myelinating Schwann cells and C fibres in the PNS.

1.4.4. Lactate metabolism in peripheral nerves

It is clear that as in the CNS, glycogen is present in the glial cell and is used as a source of energy by the neurones under certain conditions such as aglycemia, thus it is likely that this glycogen is metabolised within the Schwann cells to lactate and transferred to the axons, implying the presence of specific enzymes and transporters (namely MCTs) akin to the CNS. In comparison to the CNS little is known about the expression of MCTs in the PNS, however a recent paper has described the presence and specific localisation of MCT isoforms within peripheral nerves, including mouse sciatic nerve (Domenech-Estevez et al 2015). Investigations into the presence of MCTs using PCR revealed the expression of MCT1, 2 and 4 throughout development, with MCT1 displaying a particularly increased expression during myelination (P10) (Domenech-Estevez et al 2015). Using immunohistochemistry the specific cell type expression of MCTs was then determined; MCT1 was found to be expressed on the perineurium (Takebe et al 2008), endoneurium, Schmidt-Lanterman incisures and the paranodal region, while MCT4 was present on the endoneurium and in the myelinating Schwann cell cytoplasm, particularly the Cajal bands, outer mesaxonal line and abaxonal region (Figure 1.4.4.i) (Domenech-Estevez et al 2015). Altogether this evidence implies a more complex lactate pathway in the PNS compared to the apparent cell specific expression of a particular MCT isoform in the CNS (Domenech-Estevez et al 2015). However, due to the finding that myelinating Schwann cells display a specific pattern of expression of MCT1 and MCT4 suggests an important role of the Schwann cells in supplying lactate to the axons (Domenech-Estevez et al 2015). Further investigations involving lactate biosensors provide additional support for a metabolic role of Schwann cells. Simply placing a lactate electrode against the mouse sciatic nerve whilst it was perfused with 10mM glucose aCSF revealed a constant lactate recording of about 118 μ M, suggesting lactate is continuously released from the myelinating Schwann cells under normoglycemic conditions (Brown et al 2012).

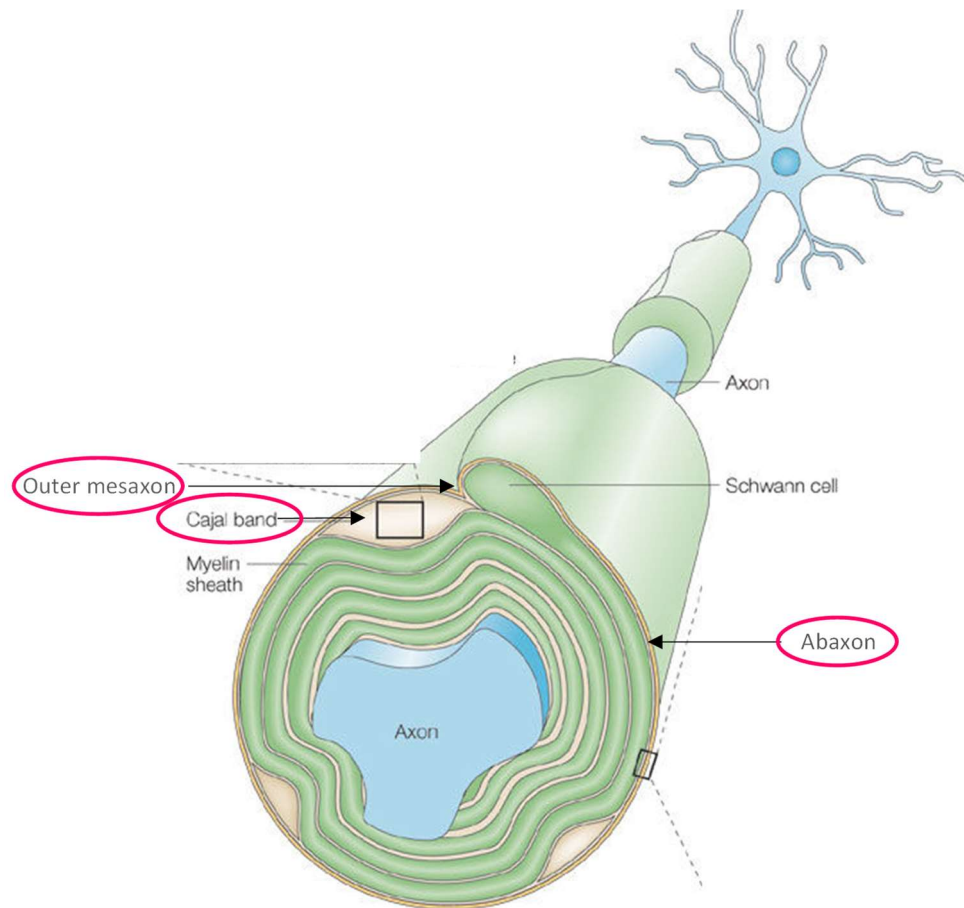


Figure 1.4.4.i: Sites of MCT4 expression on Schwann cells. Cajal bands are regions of Schwann cell cytoplasm, forming non-compact myelin, underneath the outer (abaxonal) Schwann cell membrane. The outer mesaxon is the point at which two outer Schwann cell membranes meet as a result of myelination. (Modified from Heller et al 2014).

Again, these findings do not provide direct, functional evidence that lactate supplied by myelinating Schwann cells can be used by peripheral axons as an energy substrate. Brown et al (2012) expanded on investigations into glycogen to determine whether, as in the CNS, it is actually glycogen-derived lactate that supports axon conduction. Measurements of the CAP in combination with lactate biosensor recordings showed a rapid fall in the lactate recording during aglycemia implying that the available lactate is taken up by the A fibres and oxidatively metabolised in order to maintain their conduction (Figure 1.4.4.ii) (Brown et al 2012). However, interestingly they also found that switching the aCSF supplied to the sciatic nerve from 10mM glucose to 20mM lactate resulted in support of both the A and C fibre CAP almost as well as 10mM glucose, and

perfusion of 20mM lactate after aglycemia enabled recovery of the CAP of both the A and C fibres, indicating that C fibres are able to effectively use lactate as energy substrate to support conduction despite their lack of available glycogen-derived lactate (Brown et al 2012).

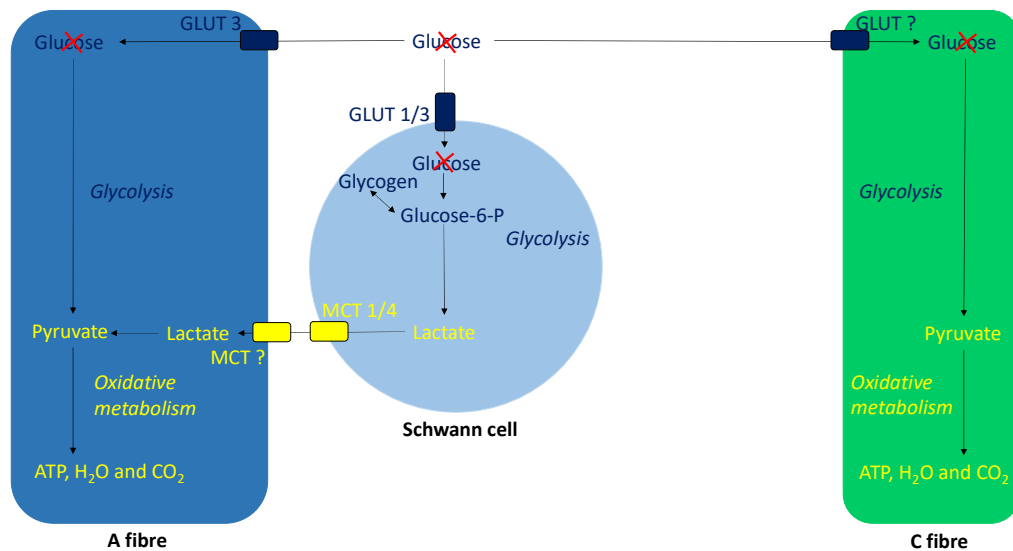


Figure 1.4.4.ii: The metabolic interaction between Schwann cells and axons in the peripheral nervous system. Schwann cells provide A fibres, but not C fibres, with glycogen-derived lactate particularly in the absence of glucose (red crosses).

Due to the universal theory that so far appears to exist between astrocytes and axons in the CNS, and Schwann cells and A fibres in the PNS, it could be inferred that the signal that stimulates glycogen-derived lactate transfer from Schwann cells to A fibres is K⁺. However, differences between the PNS and CNS in terms of K⁺ accumulation and dissipation, are significant in that the K⁺ concentration in the ECS of the sciatic nerve as a result of neuronal activity is much lower and removed at a much slower rate than in CNS tissue; indicative of diffusion in comparison to active energy dependent removal in the CNS by the respective glial cell (Hoppe et al 1991). This difference is thought to be related to the much larger ECS of the sciatic nerve compared to that of the CNS (Hoppe et al 1991). However, investigations using small and large squid axons, despite being non-mammalian, have revealed that glutamate, not K⁺, mediates the communication

between axons and Schwann cells (Lieberman et al 1989). Using antagonists of glutamate and nicotinic acetylcholine (ACh) receptors Lieberman et al (1989) showed that glutamate released by axons acts on non-NMDA glutamate receptors expressed by Schwann cells. This stimulates Schwann cells to release ACh which subsequently acts on nicotinic autoreceptors, resulting in Schwann cell hyperpolarisation (Lieberman et al 1989). Moreover, using frog sciatic nerve it has been shown that NH_3 is released by the sciatic nerve as a result of neuronal activity (Tashiro 1922), suggesting NH_4^+ may also play a role as a metabolic signal between Schwann cells and axons in the PNS. Overall it appears that the suggested mechanisms of metabolic signalling between axons and glial cells are similar in both the central and peripheral nervous system, with K^+ as the most universal however further research is still required.

1.4.5. Fructose metabolism in peripheral nerves

Fructose metabolism in peripheral nerves is of particular interest due to the link with the metabolic disorder diabetes. As an intervention of glycemic control diabetic patients can be advised to use fructose as a sweetener as in small quantities it can help reduce blood glucose levels by promoting glucose uptake and glycogen synthesis in the liver (Havel 2005). However, again due to the increasing dietary intake as well as increased flux of glucose through the polyol pathway during diabetes (Obrosova 2009), high levels of fructose may have negative effects on diabetes including diabetic neuropathy (Havel 2005).

Little is known about the ability of peripheral nerves to use fructose as an energy substrate, or the possible cell-to-cell signalling that is required, but evidence is beginning to emerge. Again for the nerve to use fructose as an energy substrate transporters and enzymes specific for this metabolism must be present. Initial Western blot analysis of the rat sciatic nerve indicated the presence of the fructose transporter, GLUT5, with further immunohistochemical investigations revealing its strong expression by axons and weak expression by Schwann cells, implying both cell types possess the ability to take up fructose (Asada et al 1998). Furthermore, the key enzymes that contribute to the polyol pathway, sorbitol dehydrogenase and aldose reductase, are expressed by Schwann cells

(Maekawa et al 2001) indicating the ability of Schwann cells to convert glucose to fructose. Despite this, if and how fructose is supplied to A fibre axons by Schwann cells, and whether fructose can be used by the C fibres is unknown. Moreover how the axons then metabolise fructose i.e. whether fructokinase is expressed or not is also unknown. However, an older study revealed the presence of fructose-1-phosphate in sciatic nerves of rats (Poulsom et al 1983) indicative of fructose metabolism by fructokinase, but despite this the precise location of fructokinase is still not clear.

A recent study has investigated the optimal substrates and their respective concentrations that can be utilised by the rat sciatic nerve during both the presence and absence of oxygen (anoxia) (Stecker & Stevenson 2015). This study provides functional data on the efficacy of different energy substrates, in the form of the nerve action potential (NAP), one of which was fructose supplied at low (5.5mM) and high concentrations (55mM) (Stecker & Stevenson 2015). When the nerve was continuously oxygenated higher (55mM) concentrations of fructose were required to maintain the NAP for the 16 hour experimental period, whilst during intermittent periods of anoxia 5.5mM fructose was more efficient at sustaining the amplitude of the NAP (Stecker & Stevenson 2015). This clearly indicates that anoxia influences the optimal substrate conditions required to maintain axon conduction, but most importantly that fructose is a sufficient energy substrate for use by the sciatic nerve to support axon conduction. However, due to the measurement of conduction obtained i.e. the NAP, the study only provides information regarding the myelinated A fibres not the unmyelinated C fibres (Stecker & Stevenson 2015).

1.5. Aim and hypotheses

The aim of my MRes was to investigate the glia-axon metabolic interactions of the mouse sciatic nerve, with a particular focus on fructose metabolism. The intention was to expand the little information already available about PNS tissue and compare it to the established knowledge about such interactions of CNS tissue to provide insight into a possible universal theory of metabolic interactions between glial cells and axons.

Firstly, I hypothesised that A fibre conduction would be better maintained than the C fibres during no energy substrate (i.e. aglycemia) conditions, confirming the previous findings by Brown et al (2012) that A, not C, fibres benefit from Schwann cell glycogen-derived lactate. To further investigate the metabolic properties of A and C fibres, metabolic inhibitors, iodoacetate (blocks glycolysis) and cyanide (blocks oxidative phosphorylation) were used. I hypothesised that iodoacetate supplied with 10mM glucose (glycolytic) would reduce A and C fibre CAP maintenance compared to no energy substrate but in combination with 20mM lactate (oxidative) conduction would be maintained. I also hypothesised that the presence of cyanide would result in rapid failure of both the A and C fibre CAP.

Based on knowledge that the A fibres benefit from Schwann cell glycogen-derived lactate but the C fibres do not, I hypothesised that the C fibres would metabolise fructose directly whilst A fibre fructose metabolism would require cooperation with Schwann cells in the form of fructose-derived lactate, based on the findings by Izumi and Zorumski (2009). To investigate the role of lactate in A fibre fructose metabolism a lactate transporter blocker, α -Cyano-4-hydroxycinnamic acid (4-CIN), was used to prevent the transfer of lactate from the Schwann cells to the axons in the presence of fructose. I hypothesised that fructose supplied in the presence of 4-CIN would reduce the maintenance and recovery of the A fibre CAP but have little/no effect on the C fibre CAP. Lactate biosensors were also used to investigate lactate. These were incorporated into the electrophysiology recovery experiments in order to record lactate released from the sciatic nerve into the surrounding bath solution. Finally, findings from

electrophysiology maintenance and recovery experiments in the presence of fructose led to investigations into fructokinase expression, using immunohistochemistry, of the sciatic nerve which was hypothesised to be expressed by the C, but not the A fibres.

Chapter 2: Methodology

2.1. Sciatic nerve

Male, 26-28g CD-1 mice (Charles River Laboratories International, Inc) were sacrificed by cervical dislocation and subsequent decapitation (confirmation of death) in accordance with the UK Home Office guidelines of Schedule 1. Each mouse was pinned out prone (Figure 2.1.A), partially skinned to expose the hind region and incisions were made along the hind leg and up along the spinal cord in order to expose the sciatic nerve (Figure 2.1.B). The nerve was then gently freed from surrounding tissue, taking care not to stretch, and cut just as it leaves the spinal cord (L4-6), and where it branches into the tibial, peroneal, cutaneous and sural nerves in the lower leg ensuring, the longest length of nerve possible was retrieved (Figure 2.1.C). The sciatic nerve from each hind leg was removed i.e. 2 nerves per mouse.

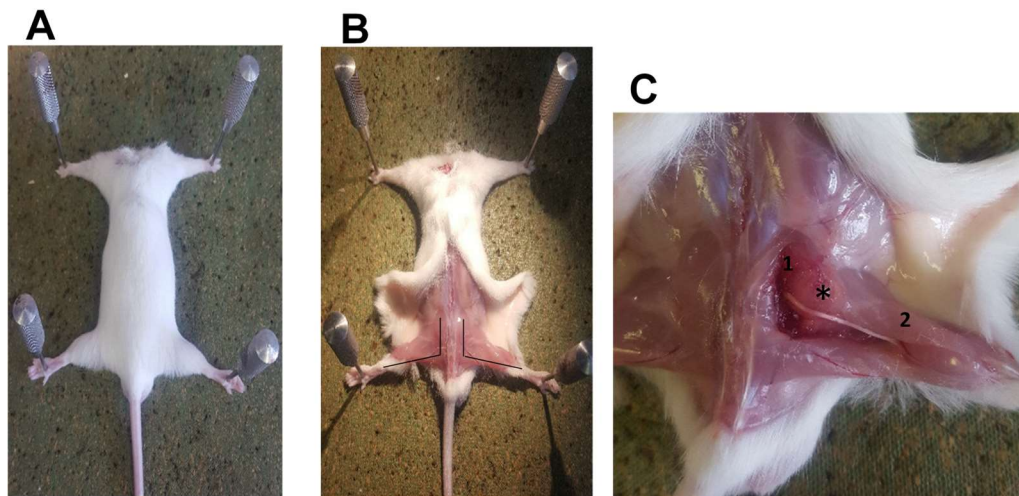


Figure 2.1: Mouse sciatic nerve dissection. **(A)** The mouse was killed in accordance with the UK home office guidelines and pinned out prone. **(B)** The mouse was skinned and incisions were made along each hind leg and up the spinal cord (black lines). **(C)** The sciatic nerve (*) was exposed, freed from surrounding tissue and cut where it leaves the spinal cord (1) and branches (2).

2.2. Electrophysiology

Each sciatic nerve was placed in an interface superfusion chamber, superfused with 10mM glucose (normoglycemic conditions) aCSF (126mM NaCl, 3mM KCl, 2mM MgSO₄, 26mM NaHCO₃, 1.25mM NaH₂PO₄ and 2mM CaCl₂) buffered at pH 7.45, maintained at 37°C, with both the artificial cerebrospinal fluid (aCSF) and chamber continuously bubbled with 95% oxygen 5% carbon dioxide gas mixture. Before the nerve was placed in the suction electrodes it was trimmed at both ends, as doing so ensured a stable recording, and the electrodes were filled with aCSF devoid of any energy substrate to remove any source of substrate reservoir available to the nerve. One end of the sciatic nerve was then captured by the stimulating electrode and the other by the recording electrode (Figure 2.2.A). Due to nerves consisting of many axons a population response for each fibre subtype (A and C) was recorded; known as the compound action potential (CAP).

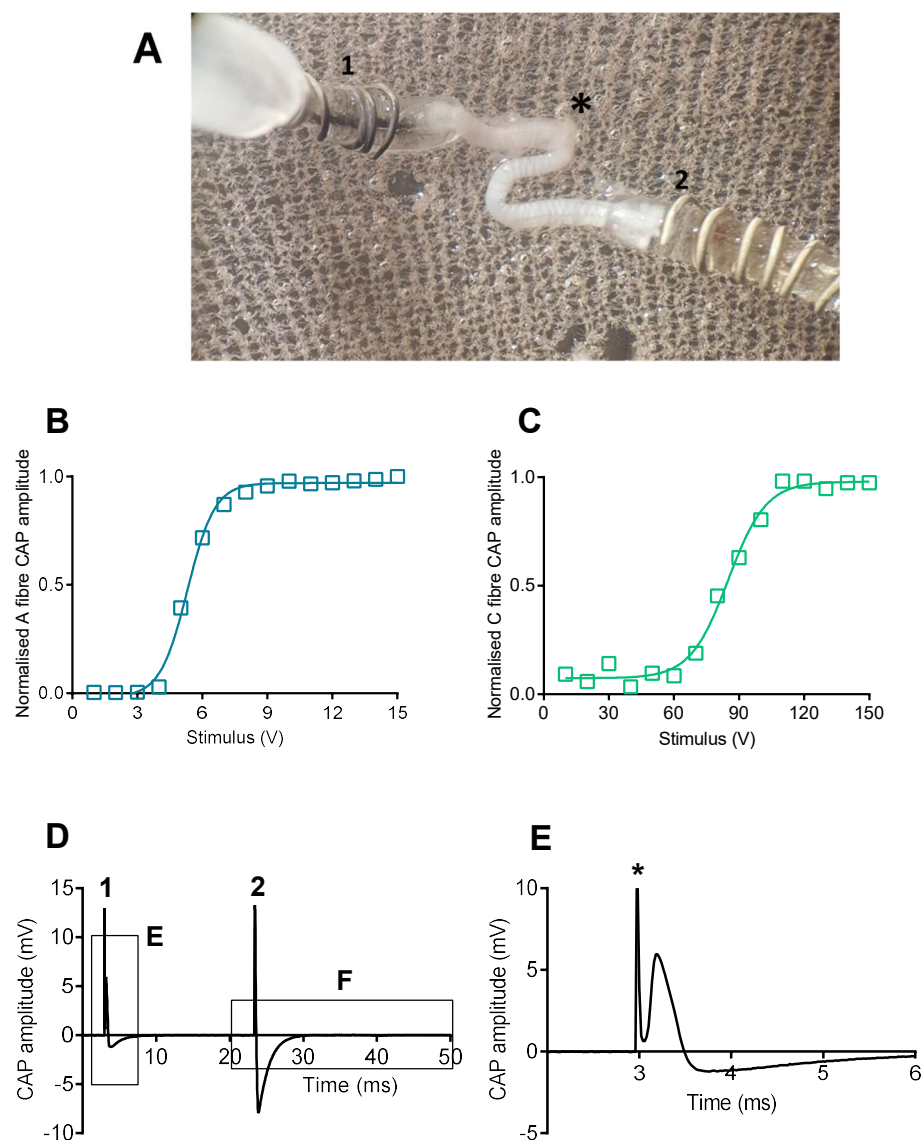
In order to record both the A and C fibre CAP simultaneously a double pulse stimulator (Grass S88) was employed (20ms delay between the two stimulations) due to the difference in threshold for recruitment of the A and C fibres. A fibres are myelinated and have a large axon diameter thus they conduct quickly and require low level stimulation in order to reach threshold (Bear et al 2007). The C fibres on the other hand are unmyelinated and are small in diameter resulting in much slower conduction and greater level of stimulation to reach threshold (Bear et al 2007). In order to determine the appropriate stimulus voltage for each fibre type a stimulus-CAP response was conducted (Figure 2.2.B and C). The voltage at which the A fibre CAP response appears to reach maximum is 10V (Figure 2.2.B) in contrast to the C fibre at 110V (Figure 2.2.C), thus supramaximal voltages (15-20V and 150V, respectively, to recruit all available A and C fibres) were used throughout experiments to ensure the CAP recorded was maximal. Clampex (Axon software) was used to generate the protocol to trigger the stimulus (1Hz frequency of stimulation i.e. once a second) and to record the CAP responses (Figure 2.2.D, E and F). The CAP was amplified by x500-1000 (Standford Research Systems preamplifier, SP560), filtered at 10-30kHz and sampled at 20kHz (Clampex, Axon software).

As shown in figure 2.2.G the C fibre CAP is much smaller than the A fibre CAP (approximately 100x; 50 μ V vs 5-6mV, respectively) thus is more affected by random electrical noise. To reduce the noise of the C fibre recordings, 60 traces i.e. one every second were averaged, a common method used to reduce the signal to noise ratio.

The nerves were equilibrated under these normoglycemic, low frequency stimulation (1Hz) conditions in order to generate a stable baseline recording of both the A and C fibre CAP before they were subjected to no substrate (no exogenously supplied energy substrate), different energy substrates of different concentrations, high frequency stimulation (HFS), metabolic inhibitors or a monocarboxylate (MCT) inhibitor. The energy substrates used included glucose (10mM), lactate (20mM) and fructose (5, 10 and 20mM), and HFS was defined as either 50Hz or 100Hz. During HFS only the A fibre CAP could be recorded due to the slow conduction velocity of the C fibres. There is about a 15ms delay after the stimulus is applied and the appearance of the C fibre CAP whereas the A fibre CAP occurs within 1ms thus a frequency of stimulation greater than every 15ms ($\sim$ 70Hz) will not allow enough time for full activation and recovery of the voltage-gated Na^+ channels present on the C fibres, preventing the recording of the CAP. Metabolic inhibitors used included 1mM sodium iodoacetate (Sigma-Aldrich), 1mM sodium cyanide (Sigma-Aldrich) and 200 μ M α -Cyano-4-hydroxycinnamic acid (4-CIN) (Sigma-Aldrich), which were added to the appropriate aCSF. 4-CIN was used to inhibit lactate transport via MCTs expressed on cell membranes at a concentration of 200 μ M in order to prevent blockage of the MCTs present on mitochondria (Choi et al 2012).

The voltage-gated Na^+ channels require sufficient time to recover from inactivation as a result of the repolarisation phase of the action potential, thus the frequency of stimulation that does not provide sufficient time for voltage-gated Na^+ channel inactivation recovery first had to be established before experiments involving HFS were conducted. This was determined using a double pulse protocol in which one pulse was applied to stimulate the A fibre CAP followed by a second at decreasing intervals (10-1ms) i.e. increased frequency of

stimulation (Figure 2.2.H). The amplitude of the first CAP was compared to the second CAP for each interval and the time at which the second CAP amplitude was smaller than that of the first CAP amplitude denotes the frequency at which there is insufficient time for recovery of voltage-gated Na^+ channel inactivation (Figure 2.2.I). 200Hz (5ms) was found to be the highest frequency of stimulation thus we can be confident that during HFS at 50Hz or 100Hz a fall in the CAP is the result of energy depletion rather than failure of the voltage-gated Na^+ channels to recover (Figure 2.2.I).



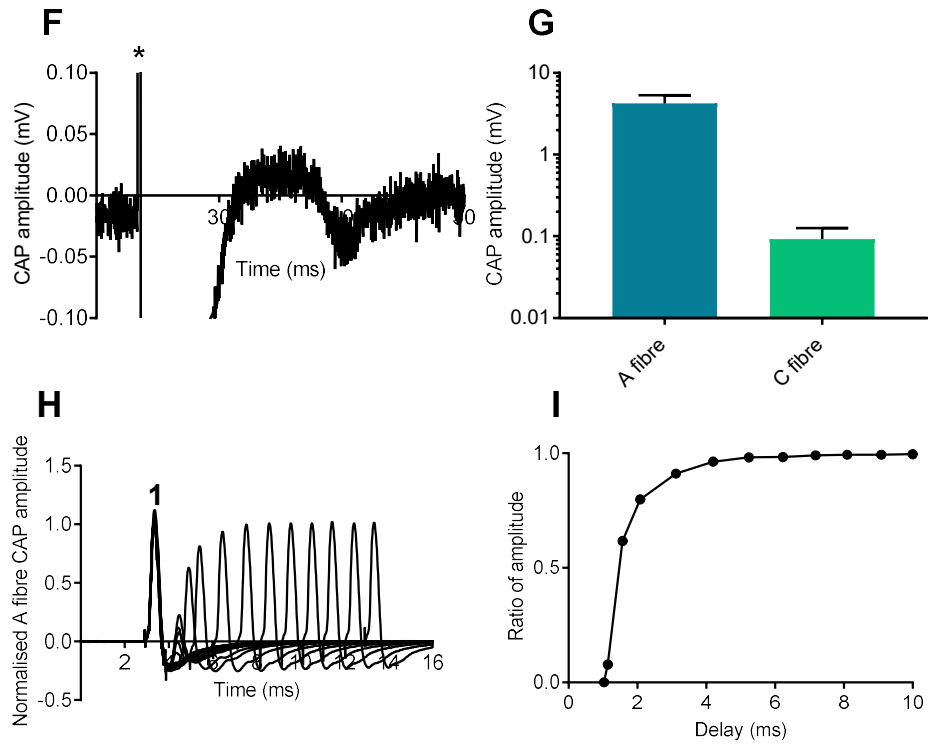


Figure 2.2: In vitro mouse sciatic nerve recordings. **(A)** The sciatic nerve (*) captured by the stimulating **(1)** and recording **(2)** suction electrodes back filled with aCSF. **(B)** A fibre stimulus response curve. CAP response peaks at ~10V. **(C)** C fibre stimulus response curve. CAP response peaks at ~110V. **(D)** Full CAP trace. Initial stimulus **(1)** recruits the A fibres **(E)**, and the second stimulus **(2)** ~20ms later recruits the C fibres **(F)**. **(E)** A fibre CAP. The CAP occurs within 1ms of the stimulus artefact (*). **(F)** C fibre CAP. The C fibre CAP is noisy and occurs after ~15ms of the stimulus artefact (*). **(G)** The amplitude of the A fibre CAP is much larger than that of the C fibre CAP (n=5 each). **(H)** Double pulse protocol. The A fibre is stimulated once **(1)** and then again at decreasing intervals (left to right). **(I)** Double pulse protocol. Reducing the delay between the first and second stimulus results in a reduction in the ratio between the amplitude of the first and second A fibre CAP below ~5ms (n=1).

2.3. Lactate biosensors

Lactate biosensors (Sarissa Biomedical) are miniature enzyme electrodes that enable real-time recording of lactate due to their selective permeability to lactate only. Once lactate enters the sensor it is oxidised to hydrogen peroxide by the enzyme lactate oxidase (Pinnacle technology 2017). Hydrogen peroxide is then detected by the platinum electrode through oxidation resulting in the recording of a signal (Pinnacle technology 2017). When used with the sciatic nerve extracellular lactate i.e. lactate released from the nerve into the surrounding bath solution, is recorded.

Lactate biosensors (500 μ m long and 50 μ m wide) were placed in the bath under the same baseline conditions as electrophysiology experiments and before use were calibrated in 1mM, 500 μ M, 200 μ M, 100 μ M and 50 μ M lactate aCSF (Figure 2.3.A and B) to ensure an increased lactate signal was recorded with increased lactate concentration. All lactate biosensor signals were recorded using Axoscope (Axon Software), converted to an axon text file (ATF) and displayed in Excel (Microsoft Office Software).

Dose-response curves for lactate released by the sciatic nerve were determined in the presence of a range of concentrations for both glucose and fructose. The lactate biosensor was first placed in the bath and superfused with 10mM glucose aCSF in order to record a stable, baseline lactate signal. 10mM glucose aCSF was then replaced with 100 μ M lactate aCSF to enable extrapolation of the concentration of lactate released from the sciatic nerve under different glucose/fructose concentrations. Once this was established aCSF was swapped back to 10mM glucose and the nerve placed over the electrode. The nerve was then superfused with glucose/fructose in descending concentration; glucose: 10, 5, 2, 1, 0.5 and 0mM, fructose: 30, 20, 10, 5, 2, 1, 0mM.

During electrophysiology experiments involving lactate biosensors the CAP was first recorded under normoglycemic conditions until stable, the lactate biosensor was then introduced into the chamber upstream of the nerve in order to record a stable, baseline lactate signal (Figure 2.3.C). The sciatic nerve was then placed

over the electrode, allowing recording of the extracellular lactate released from the nerve (Figure 2.3.D). A lactate signal rather than a lactate concentration was recorded during these experiments as a result of not calibrating the lactate electrode in 100 μ M lactate prior to exposure to the sciatic nerve. Lactate biosensor recordings could not be conducted whilst the nerve was perfused with 20mM lactate aCSF as this overwhelming concentration of lactate would interfere with the lactate released from the nerve.

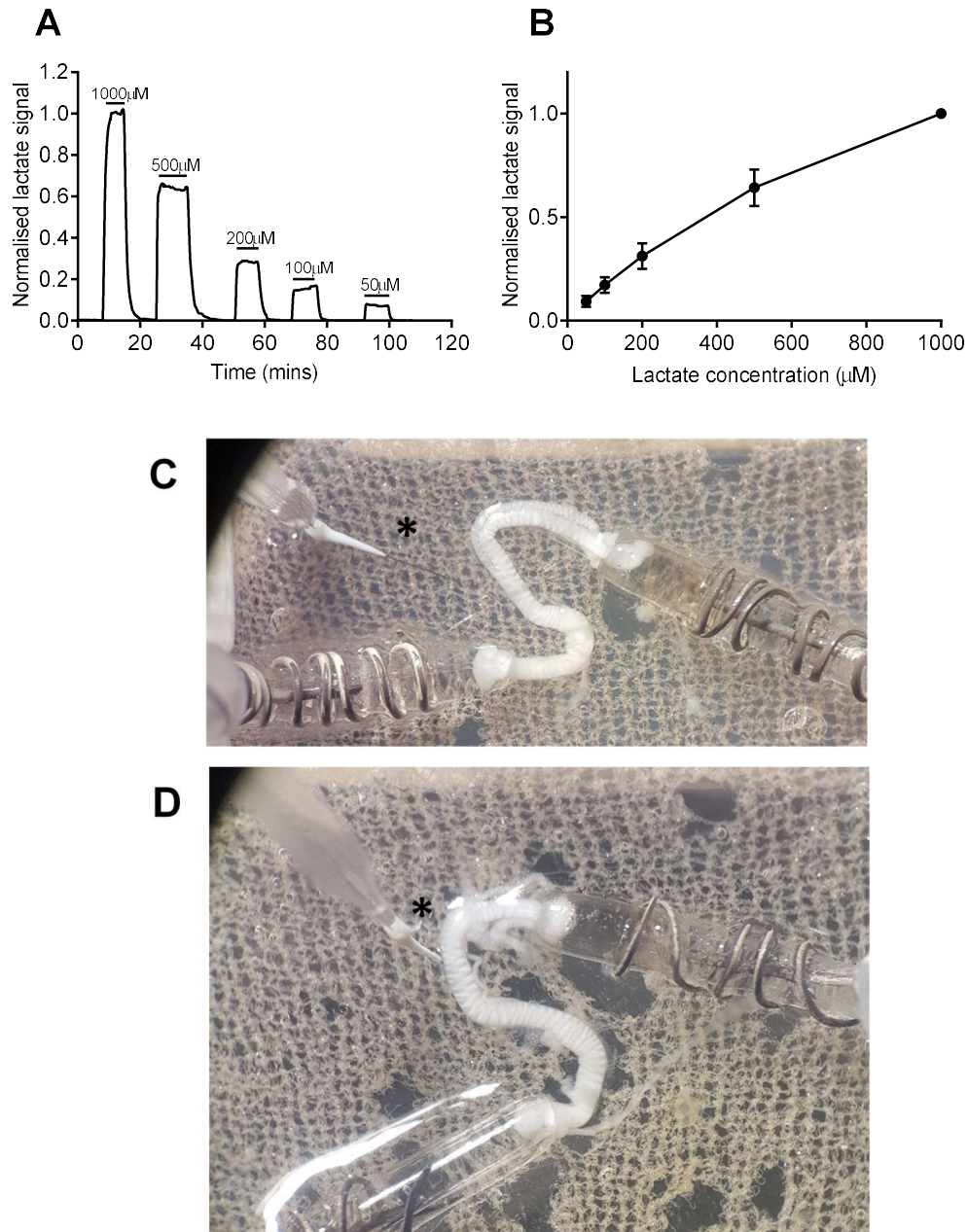


Figure 2.3: Lactate biosensor recordings. **(A)** Lactate electrode calibration. The lactate biosensor signal decreases with decreasing concentration (1000-50 μM) of superfused lactate. **(B)** Lactate calibration curve. The lactate electrode signal increases in response to increased superfused lactate concentration (n=4). **(C)** Lactate biosensor (*) is placed upstream of the sciatic nerve in order to establish a baseline lactate recording prior to the experiments. **(D)** Sciatic nerve placed on top of the electrode (*) allowing lactate released from the nerve to be recorded.

2.4. Data analysis

All data was analysed using Clampfit 10.2 (Axon software). The A fibre CAP was measured in terms of its peak amplitude relative to baseline while the C fibre was measured in terms of the amplitude between the positive and negative peaks or its area. Lactate biosensor recordings and the CAPs were then normalised to 1 under normoglycemic conditions and curve fitting (Boltzmann-sigmoidal function) was performed with Microsoft Excel. In order to quantify and statistically compare data, the area under the curve (AUC) of the normalised CAP trace was calculated (Graphpad Prism) for a maximum of 8 hours from the onset of the experimental condition or once the CAP had fallen to zero if this occurred before 8 hours. Curve fitting was used to extrapolate the data of falling CAP traces that did not fall to zero within 8 hours (Figure 2.4). AUC (normalised CAP x minutes (NCAP.mins)), rather than latency to failure (as reported in prior related papers), was calculated due to the finding that certain conditions did not result in CAP failure but maintained the CAP fully for the 8 hour experimental period, thus is used to quantify and compare CAP maintenance. CAP recovery experiments were quantified as percentage recovery and the time taken for the CAP to start recovering, determined from curve fits, and the lactate electrode slope (normalised lactate signal x minutes (Nlactate signal.mins)) recordings fitted with linear regression (Graphpad Prism). Statistical analysis was performed with Graphpad Prism (ns $P > 0.05$, * $P \leq 0.05$ ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$) where the n number relates to the number of nerves and included one-way ANOVA with Dunnett's post hoc test (comparisons to control) or one-way ANOVA with Sidak's post hoc test (selected comparisons) when comparisons were made between 3 or more conditions and unpaired two-tailed t-test when comparisons were made between 2 conditions. One-way ANOVA was used to make comparisons within each fibre type whilst unpaired t-tests were used to make the occasional comparison between fibres types. Experiments were excluded from analysis if CAP failure appeared to result from nerve damage rather than the experimental condition.

A representative trace (i.e. 1 nerve) from each experimental condition is displayed, all values are expressed as mean $\pm$ standard deviation (SD) and in general blue bars= A fibre, green bars= C fibre.

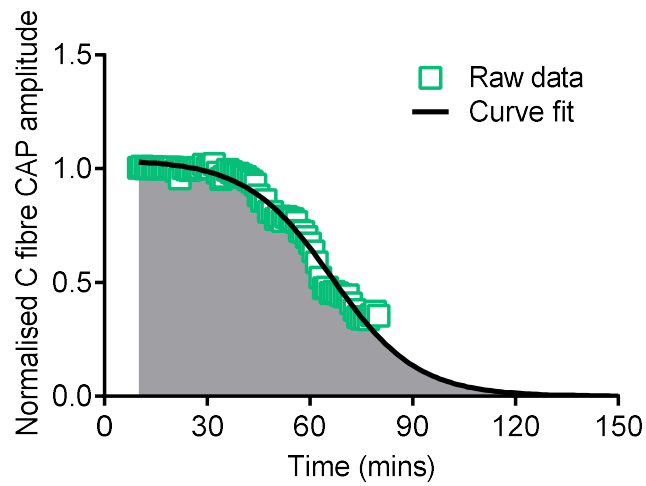


Figure 2.4: Curve fitting of CAP trace allowing extrapolation of the data down to zero for AUC analysis (grey shading).

2.5. Western Blotting

Both sciatic nerves of the mouse were obtained as previously described and placed in an Eppendorf tube submerged in ice. In addition to sciatic nerve, liver was also extracted and processed in the same way to act as a positive control for the presence of fructokinase. Each Eppendorf was weighed and the weight of an empty Eppendorf subtracted in order to determine the weight of the tissue which was then used to calculate the volume of lysis buffer added (1ml per 100mg of tissue). Each sample was then homogenised on ice using a probe homogeniser and spun on a daisy spinner for 20 minutes at 4°C. Finally the samples were spun on a bench top centrifuge for 5 minutes at 850 RCF and 4°C before the supernatant was pipetted out (pellet discarded) and SDS solubilisation buffer added (6x dilution).

To ensure the same concentration of total protein for each tissue type was loaded on to the Western blot gel a standard protein concentration curve had to first be established allowing the total protein concentration of the sciatic nerve and liver to be extrapolated. This is known as protein estimation and was conducted using the Lowry test. Bovine serum albumin (BSA) was used as the standard protein and made up in increasing dilutions with distilled water (11 dilutions made up to 200µl starting at largest dilution of 0µl BSA and 200µl distilled water to the smallest dilution of 100µl each in 10µl steps). Dilutions of sciatic nerve (1:10 and 1:50) and liver (1:50 and 1:100) were also made up. 200µl of Working Lowry reagent (0.5% copper sulphate pentahydrate, 2.7% potassium/sodium tartrate and 10% sodium carbonate (anhydrous) in 0.5M sodium hydroxide) was added to all dilutions of BSA, sciatic nerve and liver and left for 10 minutes at room temperature before 100µl of Folin's reagent (1:1 Folin's reagent and distilled water) was added for another 45 minutes at room temperature. All dilutions were performed twice to enable an average recording. 100µl of each dilution and repeat was added to a 96-well plate which was subsequently placed in a plate reader and the absorbance measured at 720nm. Absorbance could then be plotted against the known concentration of BSA enabling the protein concentration of sciatic nerve and liver to be extrapolated

from the absorbance at each dilution. The protein concentration of sciatic nerve was calculated at 6.41mg/ml and the liver at 16.70mg/ml. 25µg was chosen as the total concentration of protein for each tissue loaded onto the gel (gradient 4-20%) thus the volume of sciatic nerve required was 3.9µl while the volume of liver was 1.5µl. These volumes were then doubled resulting in 8µl of sciatic nerve and 3µl of liver. 3µl of protein molecular weight marker was loaded into the first lane of the Western blot gel, in order to provide a reference for the bands of different sized proteins. Liver and sciatic nerve were loaded into the second and third lanes, respectively. 100V was then applied across the gel for 90 minutes.

After the gel had ran the proteins were transferred onto nitrocellulose membrane filters by electroblotting. This transfer is important as it allows antibodies to access the proteins for detection. The gel was first soaked in transfer buffer (1x dilution) then placed on top of filter paper on a sponge. The nitrocellulose sheet was then added on top of the gel and finally another piece of filter paper and sponge, avoiding the formation of bubbles between layers. 100V was then applied across the gel again for 60 minutes in transfer buffer (1x dilution) before the gel was dried overnight between 2 clean pieces of filter paper.

Proteins bands were initially visualised using the Ponceau red stain, reversed with distilled water, then immunoprobed for the enzyme fructokinase. The nitrocellulose was blocked in blocking buffer (5% milk powder in TBST) for 1 hour at room temperature by shaking incubation, then incubated with the primary antibody, anti-ketohexokinase chicken IgY (1:500 dilution in blocking buffer, Sigma-Aldrich), overnight at 4°C with gentle rocking. After incubation the nitrocellulose was quickly rinsed in TBST (0.1% Tween-20 in 10x TBS) then washed 3 times for 15 minutes each in TBST on the orbital shaker. Licor Odyssey IRDye 800CW donkey anti-chicken secondary IgG antibody (1:10,000 dilution in blocking buffer, LI-COR Biosciences, Ltd) was then added to the nitrocellulose and left to incubate in the dark for 1 hour at room temperature on the orbital shaker. Finally the nitrocellulose was washed 3 times in TBST for 15 minutes

each in the dark before dried between two pieces of filter paper. The nitrocellulose was then read on a Licor Odyssey reader.

2.6. Immunohistochemistry

After dissection mouse sciatic nerve and liver were snap frozen on dry ice then embedded in OCT, a cryo-embedding media. Sections of 10µm thick were cut on a cryostat and thaw-mounted onto superfrost slides, which were stored at -80°C until required. Each slide consisted of 2 liver sections, and 2 cross sections and 2 longitudinal sections of sciatic nerve. Before slides were used for immunohistochemistry they were removed from the -80°C freezer and allowed to warm at room temperature. A two-day immunohistochemistry protocol was employed and throughout slides were processed in a humidified chamber. Tissue sections were first fixed with 70% ethanol for 5 minutes then washed with phosphate buffered saline (PBS) twice for 5 minutes each. Buffer 1 (PBS + 1% BSA + 0.3% Tween-20) was then used to wash the sections another 3 times for 5 minutes each. The secondary antibody was raised in rabbit thus normal rabbit serum (NRS) was used as the blocker for non-specific background staining. Sections were then incubated in NRS (Buffer 1 + 2% normal rabbit serum) at room temperature for 1 hour, after which it was tapped off and the primary antibodies: anti-ketohexokinase chicken IgY, 1:200 dilution in Buffer 1 (Sigma-Aldrich) and anti-neurofilament 200 mouse IgG clone No.N52, 1:200 dilution in Buffer 1 (Sigma-Aldrich) were added and incubated overnight at 4°C. At this stage a control was performed in which no primary antibody was added but instead remained incubated in NRS blocker overnight at 4°C.

After overnight incubation the primary antibody was tapped off and the sections washed three times in Buffer 1 for 5 minutes each. The secondary antibodies: rabbit polyclonal chicken IgY (fluorescein isothiocyanate-labelled 488nm), 1:500 dilution in buffer 1 (Abcam ab6749) and rabbit anti-mouse IgG (Alexa flour 568), 1:500 dilution in Buffer 1 (Fisher Scientific) were then added to slides and incubated in the dark for 1 hour at room temperature. From here onwards care was taken to avoid subjecting sections to light, thus slides were kept in the dark as much as possible. At this point another control was performed where a slide incubated with the primary antibodies was not incubated with secondary antibodies but instead remained incubated with Buffer 1. In addition the no

primary control was incubated with secondary antibodies thus resulting in a no primary but secondary antibody control and a primary but no secondary antibody control. The sections were then washed three times in Buffer 1 and three times in PBS all at 5 minutes each. Finally the sections were briefly flooded with DAPI (stains for DNA in the nucleus, in this case the Schwann cells and hepatocytes) and washed with PBS. DABCO, an aqueous mounting medium, was applied to cover slips and slides placed on top, being careful to avoid air bubbles forming, and sealed using nail varnish. The slides were then viewed using confocal microscopy (Zeiss), at x63 for the sciatic nerve sections and x20 for liver, and processed using Fiji (ImageJ). Control slides were viewed under an epifluorescent microscope (x20) using Advanced SPOT.

Although n numbers for the electrophysiology and lactate biosensor experiments are low in some cases (as a result of the difficult recording technique) and the Western blot/immunohistochemistry has yet to be repeated on more animals, this preliminary data suggests promising results and conclusions have been made.

2.7. Experiment timeline

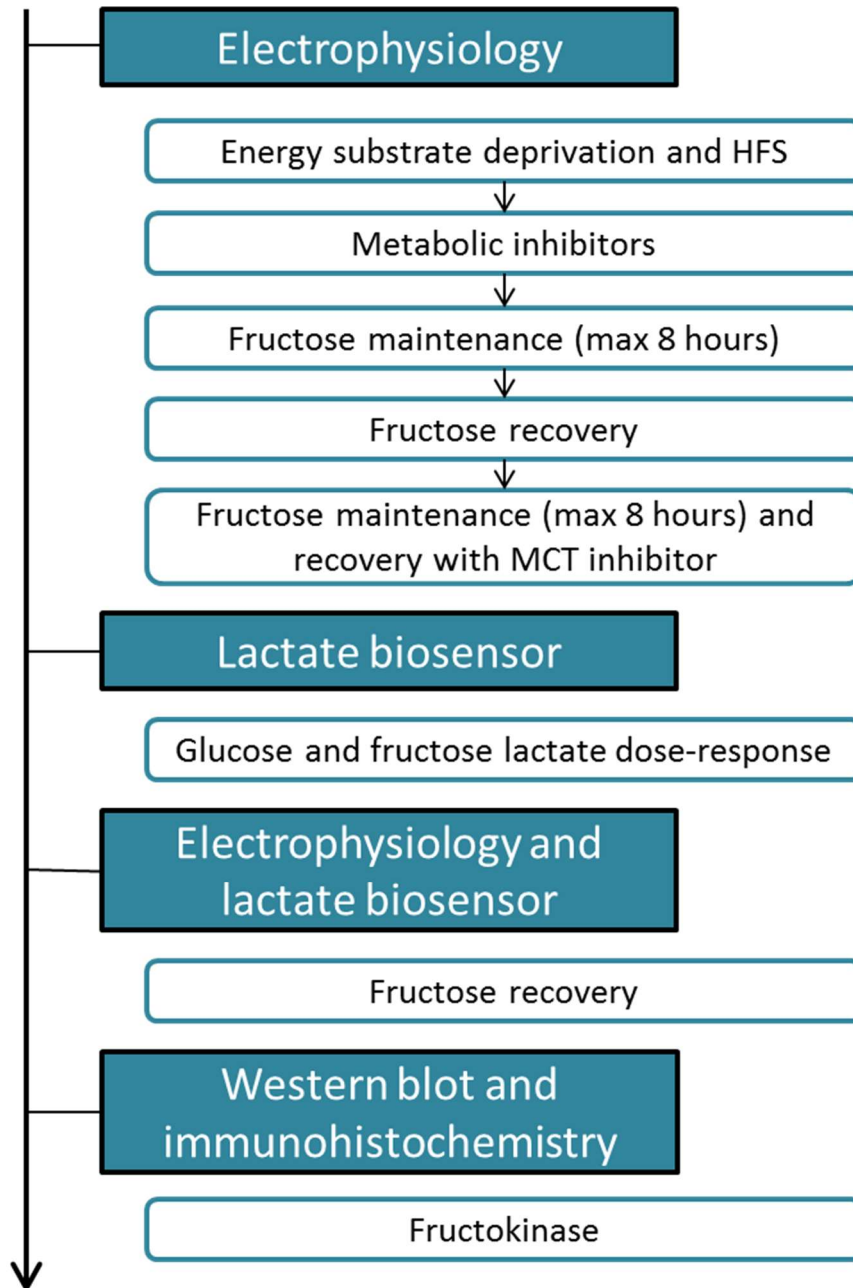


Figure 2.7: Timeline of experiments conducted between September 2016 and August 2017.

Chapter 3: Results

3.1. The effect of withdrawal of an exogenous energy substrate on A and C fibre conduction

Glucose is the principal nervous system energy substrate, which is clear from the complete maintenance of the A and C fibre CAP when the sciatic nerve was superfused with 10m glucose aCSF for the full 8 hour experimental period (Figure 3.1.A and B). The removal of an exogenous supply of energy substrate resulted in the failure of both the A and C fibre CAP, however the A fibre CAP was maintained significantly longer compared to that of the C fibre CAP; 151.3 $\pm$ 27.8 NCAP.mins vs 63.5 $\pm$ 4.4 NCAP.mins, respectively (Figure 3.1.C). This data is complementary to the previous findings by Brown et al (2012).

Due to the sufficiently long maintenance of A fibre conduction in the absence of an energy substrate, the frequency of stimulation was increased under these conditions in order to accelerate the failure and enable CAP recovery experiments to be conducted in a timely fashion. The reasoning behind this was that any energy/energy substrates that remained available and used to maintain conduction during energy substrate deprivation would be metabolised quicker, thus attenuating A fibre CAP maintenance. The standard frequency of stimulation used throughout, including the no substrate experiments described above, was 1Hz. This was then increased to 50Hz or 100Hz during A fibre CAP recovery experiments (described later). 50Hz stimulation in addition to no substrate supply significantly reduced the maintenance of the A fibre CAP to 76.8 $\pm$ 10.3 NCAP.mins, while 100Hz further reduced it to 42.7 $\pm$ 16.3 NCAP.mins (Figure 3.1.D and E).

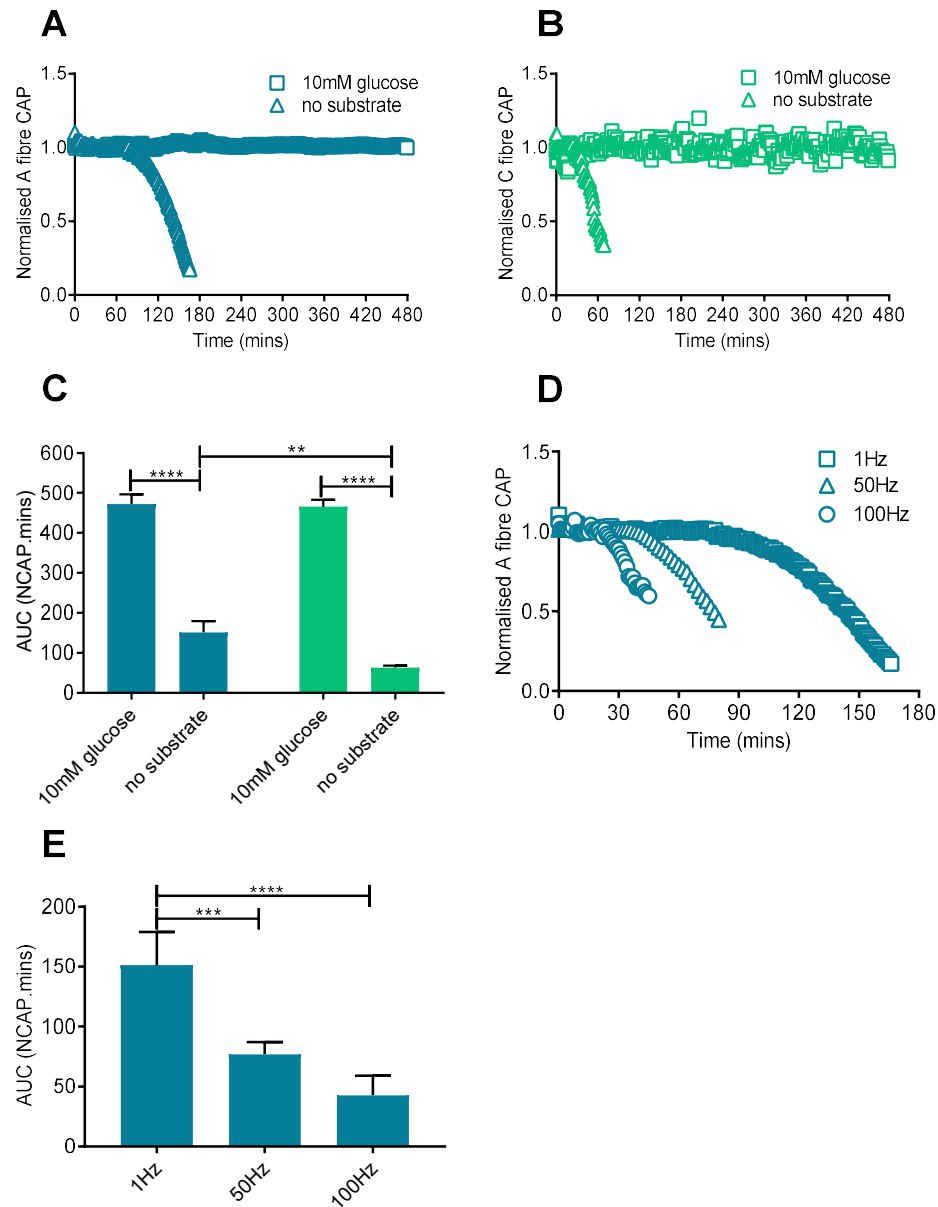


Figure 3.1: The A fibre CAP was better maintained than the C fibre CAP in the absence of an exogenous energy substrate. **(A)** 10mM glucose maintained the A fibre CAP for the full 8 hours whilst no energy substrate conditions resulted in failure of the CAP. **(B)** The C fibre CAP was fully maintained in the presence of 10mM glucose but failed in the absence of an energy substrate. **(C)** No substrate supply significantly reduced maintenance of both the A (n=4) and C (n=4) fibre CAP compared to fully maintained conduction in the presence of 10mM glucose (n=3 and n=3 respectively). The C fibre CAP was maintained significantly less in the absence of an energy substrate compared to the A fibre CAP. **(D)** Increasing the frequency of stimulation from low (1Hz) to 50Hz or 100Hz progressively reduced the maintenance of the A fibre CAP during no substrate supply. **(E)** 50Hz (n=4) and 100Hz (n=4) significantly accelerated the failure of the A fibre CAP in the absence of an energy substrate.

3.2. The effect of metabolic pathway inhibitors on A and C fibre conduction

Iodoacetate is an inhibitor of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sabri & Ochs 1971), and its presence when the sciatic nerve was supplied with 10mM glucose aCSF resulted in failure of both the A and C fibre CAP (Figure 3.2.A and B) of which was attenuated compared to no energy substrate; 44.8 +/- 5.7 NCAP.mins vs 151.3 +/-27.8 NCAP.mins (A) and 28.7 +/- 3.4 NCAP.mins vs 63.5 +/- 4.4 NCAP.mins (C), respectively (Figure 3.2.C). However maintenance of A fibre conduction appeared to be equivalent during no substrate when superfused with 20mM lactate and 1mM iodoacetate (141 +/- 21.6 NCAP.mins) (Figure 3.2.A and C) in comparison to C fibre conduction which was increased to 140.0 +/- 18.1 NCAP.mins during 20mM lactate and 1mM iodoacetate, compared to no substrate supply (Figure 3.2.B and C).

Cyanide is a potent inhibitor of complex IV of the electron transport chain (ECT), preventing the reduction of oxygen to water and thus the formation of ATP (Champe et al 2008). The addition of 1mM cyanide to 10mM glucose aCSF led to rapid failure of the A and C fibre CAP (Figure 3.2.A and B) resulting in their maintenance for only 9.2 +/- 1.9 NCAP.mins and 7.0 +/- 0.9 NCAP.mins, respectively (Figure 3.2.D).

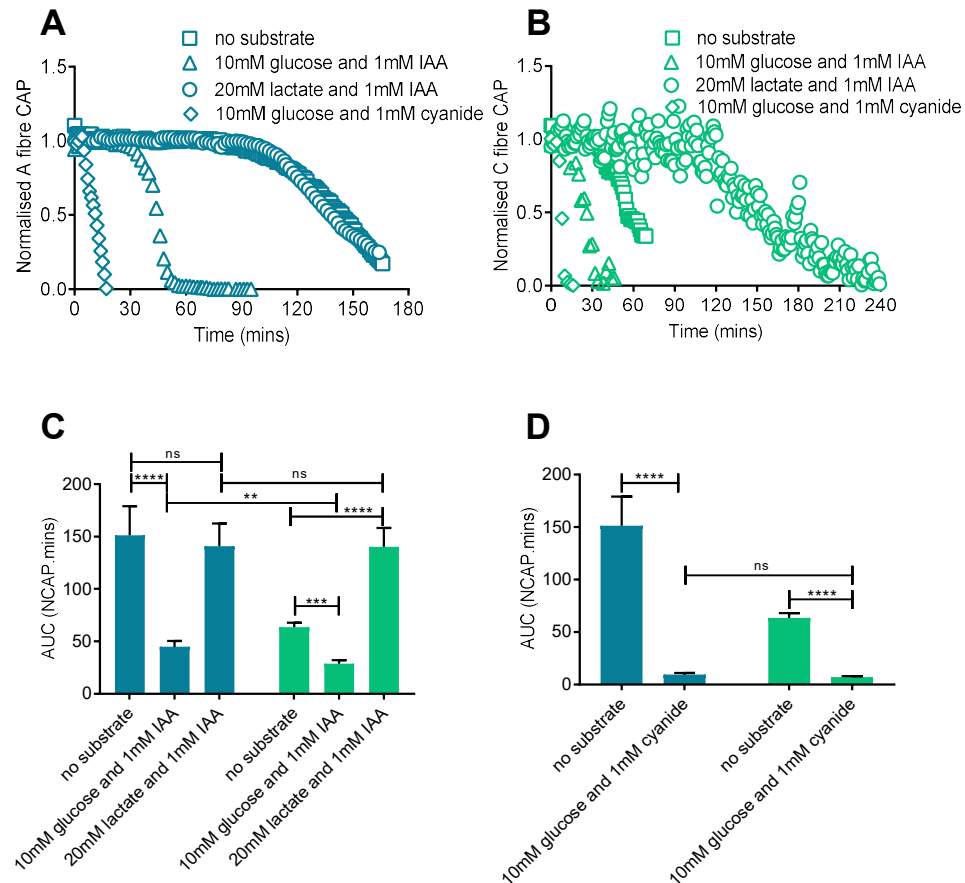


Figure 3.2: The effect of metabolic inhibitors on A and C fibre conduction. **(A)** The presence of 10mM glucose with either 1mM IAA or 1mM cyanide attenuated the maintenance of the A fibre CAP, whilst superfusion of 20mM lactate and 1mM IAA appeared to have no effect in comparison to no substrate conditions. **(B)** 1mM IAA or 1mM cyanide with 10mM glucose reduced the maintenance of the C fibre CAP compared to no substrate supply. 20mM lactate and 1mM IAA increased the maintenance of C fibre conduction compared to no energy substrate but still resulted in failure of the CAP. **(C)** 10mM glucose supplied in the presence of 1mM IAA significantly reduced the maintenance of conduction of both the A (n= 4) and C (n=4) fibres. 20mM lactate and 1mM IAA was equivalent to no substrate supply at maintaining the A fibre CAP (n=6) but significantly increased maintenance of the C fibre CAP (n=2) to that of the A fibre with 20mM lactate and 1mM IAA/no substrate. **(D)** 10mM glucose with 1mM cyanide significantly reduced the maintenance of the A (n=7) and C (n=3) fibre CAP to a similar length compared to no substrate.

3.3. The use of fructose as an energy substrate to maintain A and C fibre conduction

Investigations into fructose as a sufficient energy substrate in the sciatic nerve began with experiments to determine its ability to maintain A and C fibre conduction at different concentrations. During these experiments glucose aCSF was replaced with fructose, i.e. no exogenous glucose was supplied to the nerve, at higher (20mM) and lower (10 and 5mM) concentrations.

20mM fructose was found to be equivalent to 10mM glucose at fully maintaining the A and C fibre CAP for the 8 hour experimental period; 465.7 $\pm$ 16.4 NCAP.mins vs 472.5 $\pm$ 23.8 NCAP.mins (A) and 468.3 $\pm$ 14.8 NCAP.mins vs 465.9 $\pm$ 16.7 NCAP.mins (C), respectively (Figure 3.3.A, B and C). Maintenance of A fibre conduction was reduced to 348.6 $\pm$ 62.4 NCAP.mins when the sciatic nerve was superfused with 10mM fructose, and further reduced to that of no substrate (151.3 $\pm$ 27.8 NCAP.mins) when supplied with 5mM fructose; 202.8 $\pm$ 100.9 NCAP.mins (Figure 3.3.A and C). Similar to the A fibre CAP, maintenance of the C fibre CAP was significantly reduced in the presence of 10mM fructose (409.2 $\pm$ 3.2 NCAP.mins) and 5mM fructose (388.7 $\pm$ 7.4 NCAP.mins) (Figure 3.3.B and C). However, in contrast to the A fibre CAP, the C fibre CAP was better maintained with 5mM fructose compared to that of no energy substrate supply; 388.7 $\pm$ 7.4 NCAP.mins vs 63.5 $\pm$ 4.4 NCAP.mins (Figure 3.3.B and C).

To further investigate whether fructose was capable of maintaining A fibre conduction equally as efficiently as glucose, the sciatic nerve was first preincubated in either 10mM glucose (i.e. no substrate experiments), 20mM fructose or 10mM fructose and then deprived of an exogenous energy substrate. Preincubation in either 10mM glucose or 20mM fructose resulted in similar maintenance of A fibre conduction during no substrate supply (151.3 $\pm$ 27.8 NCAP.mins vs 155.6 $\pm$ 9.8 NCAP.mins, respectively) whilst preincubation in 10mM fructose attenuated the maintenance of the A fibre CAP to similar to that of the C fibre CAP during no substrate supply preincubated in 10mM glucose; 61.2 $\pm$ 17.7 NCAP.mins vs 63.5 $\pm$ 4.4 NCAP.mins (Figure 3.3.D and E).

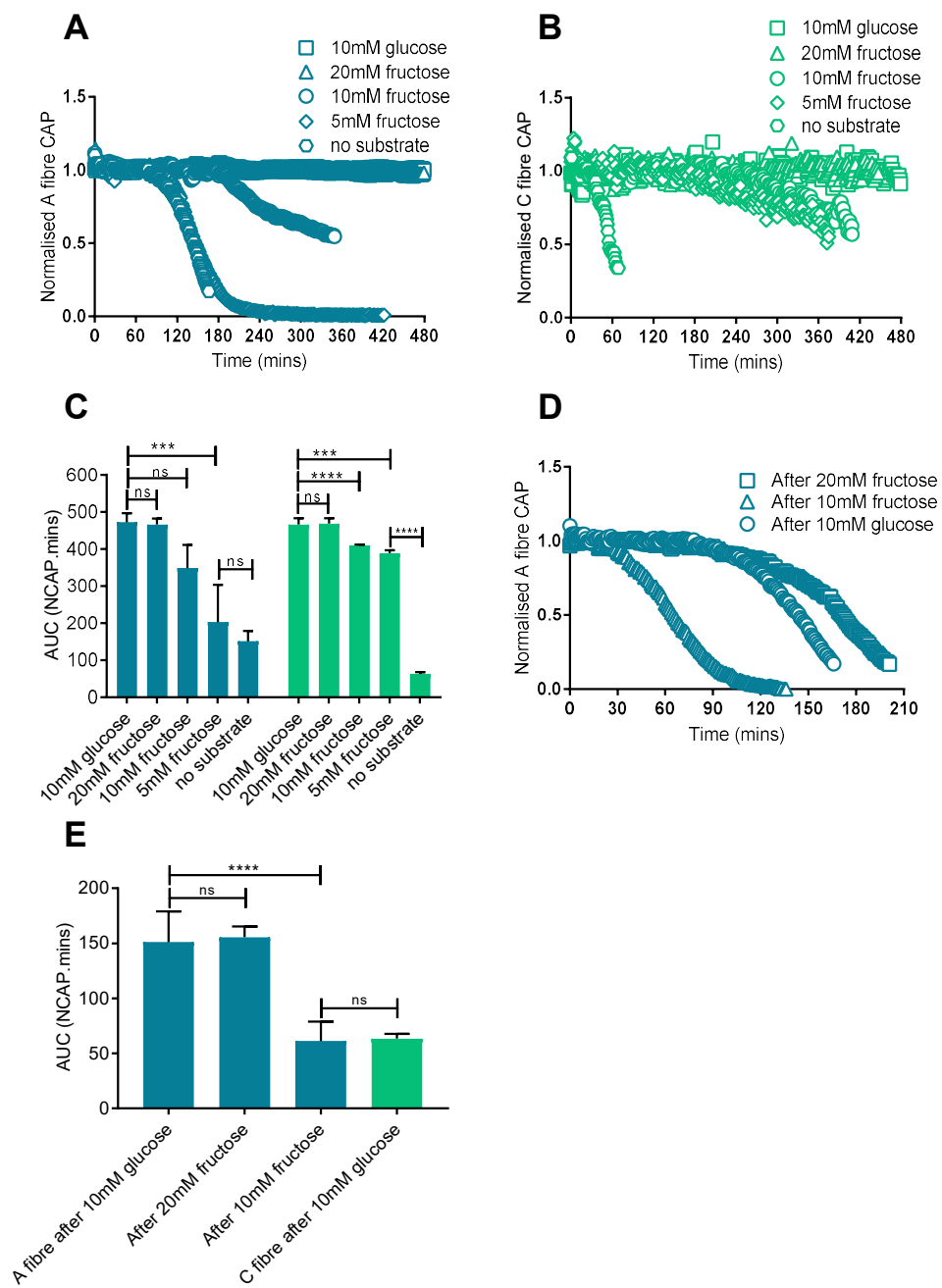


Figure 3.3: Fructose maintained A and C fibre conduction. **(A)** A higher (20mM) concentration of fructose was equivalent to maintain the A fibre CAP as 10mM glucose for 8 hours. Lower concentrations (10 and 5mM) of fructose were unable to fully maintain the A fibre CAP, in particular 5mM fructose appeared equivalent to no substrate supply. **(B)** 20mM fructose fully maintained the C fibre CAP similar to 10mM glucose while, 10mM and 5mM fructose did not but 5mM fructose maintained it for longer than no substrate supply. **(C)** There was no significant difference in the ability of 20mM fructose or 10mM glucose to maintain the A (n=3 and n=3, respectively) and C (n=3 and n= 3, respectively) fibre CAP fully. The lower the concentration of fructose (10mM n=3, 5mM n=3) the greater reduction in the maintenance of the A fibre CAP compared to 10mM glucose, in particular 5mM fructose did not significantly increase maintenance of the CAP compared to no substrate. 10mM fructose (n=3) and 5mM fructose (n=2) significantly reduced the maintenance of the C fibre CAP but 5mM fructose maintained it significantly more than no substrate supply. **(D)** Removal of an exogenous energy substrate resulted in similar maintenance of the A fibre CAP when preincubated in 10mM glucose or 20mM fructose, but preincubation in 10mM fructose reduced maintenance of A fibre CAP. **(E)** There was no significant difference in the maintenance of the A fibre CAP during no substrate after preincubation with 10mM glucose (n=4) or 20mM fructose (n=3). Compared to preincubation with 10mM glucose, 10mM fructose (n=4) significantly reduced the maintenance of A fibre conduction similar to that of the C fibre after 10mM glucose (n=4).

3.4. The use of fructose as an energy substrate to recover A and C fibre conduction

Investigations then progressed to determine whether fructose was a sufficient energy substrate to recover the A and C fibre CAP. The rationale behind this was that more energy is required to restore, rather than simply maintain the CAP. These experiments involved subjecting the sciatic nerve to no substrate conditions of which resulted in failure of the CAP. Once the CAP amplitude reached around 50% of its baseline 10mM glucose, 20mM fructose or 10mM fructose was superfused.

It immediately became apparent that 10mM glucose and 20mM fructose, but not 10mM fructose, could recover the A fibre CAP (Figure 3.4.A). Although statistically 20mM fructose recovered the A fibre CAP to less than that of 10mM glucose (87.7 +/- 11.2% and 98.7 +/- 1.9%), it demonstrates the ability of fructose to act as an energy substrate sufficient to recover A fibre conduction (Figure 3.4.C). On the other hand both concentrations of fructose were able to recover the C fibre CAP equivalent to 10mM glucose; 10mM glucose 92.2 +/- 8.8% , 20mM fructose 99.9 +/- 0.1% and 10mM fructose 93.8 +/- 7.4% (Figure 3.4.B and C).

Furthermore, a clear difference in the time taken from the addition of the energy substrate and the start of A fibre CAP recovery was evident between 10mM glucose and 20mM fructose (Figure 3.4.A). It was found that on addition of 10mM glucose CAP recovery occurred by 7.2 +/- 4.4 mins whilst in the presence of 20mM fructose it took 35.7 +/- 16 mins (Figure 3.4.D). However again in terms of C fibre CAP recovery the time taken was not significantly different between 10mM glucose, 20mM fructose and 10mM fructose; 1.3 +/- 0.6 mins, 1.7 +/- 1.2 mins and 5.7 +/- 5.7 mins, respectively (Figure 3.4.B and D).

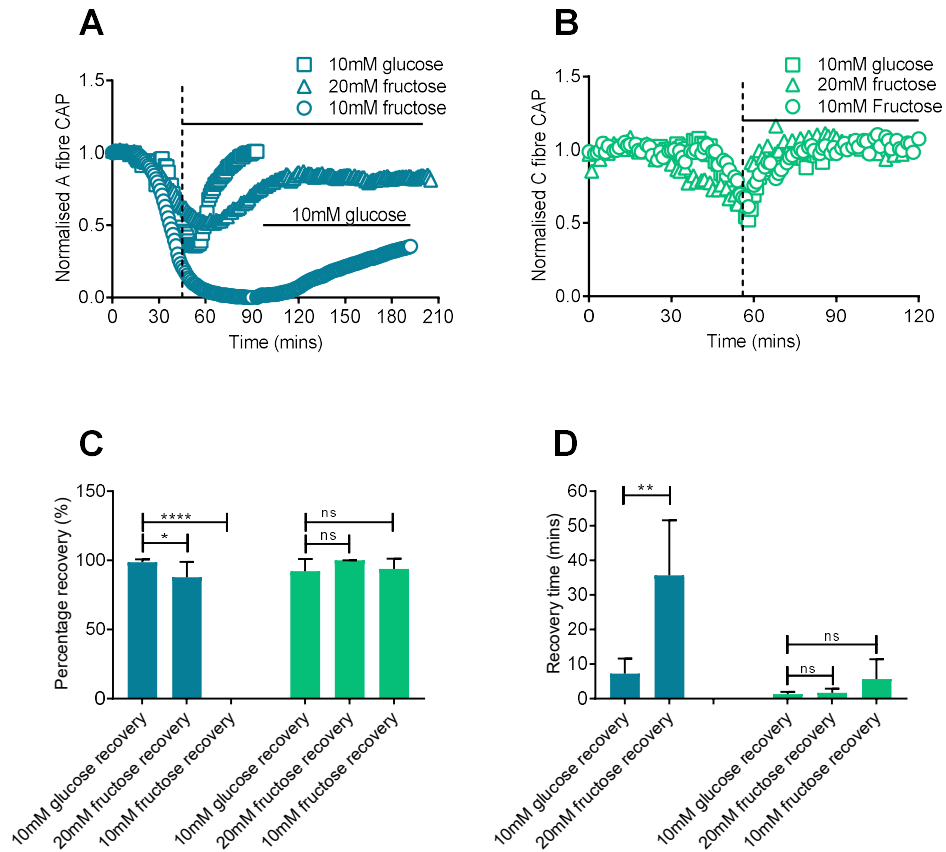


Figure 3.4: Fructose recovered A and C fibre conduction. (A) The A fibre CAP was recovered by 10mM glucose and 20mM fructose while 10mM fructose did not. **(B)** 10mM glucose, 20mM fructose and 10mM fructose recovered the C fibre CAP. **(C)** 20mM fructose (n=3), but in particular 10mM fructose (n=4), did not recover the A fibre CAP as much as 10mM glucose (n=6), whilst 10mM glucose (n=3), 20mM fructose (n=3) and 10mM fructose (n=3) were able to recover the C fibre CAP fully. **(D)** The start of A fibre CAP recovery occurred significantly later with 20mM fructose than 10mM glucose while both recovered the C fibre CAP equally quick.

3.5. The effect of 4-CIN on the use of fructose as an energy substrate to maintain and recover A and C conduction

α -Cyano-4-hydroxycinnamic acid (4-CIN) is a blocker of MCT2 (Wender et al 2000), thus it prevents the transport of lactate and was used to investigate the role lactate might play in the utilisation of fructose as an energy substrate by the A fibres.

As before, experiments concerning fructose as a sufficient energy substrate for the sciatic nerve, in the presence of 4-CIN, began with its ability to maintain the CAP. Previously it was shown that 20mM fructose fully maintained the A fibre CAP equally as well as 10mM glucose. With the addition of 200 μ M 4-CIN it was found that 10mM glucose could still fully maintain the A fibre CAP (488.5 $\pm$ 18.4 NCAP.mins) but not 20mM fructose, where the CAP showed a late failure (Figure 3.5.A). Despite this the reduction in maintenance of the A fibre CAP during 20mM fructose and 200 μ M 4-CIN was not found to be significantly different from the full 8 hour maintenance with 20mM fructose; 430.8 $\pm$ 53.2 NCAP.mins vs 465.7 $\pm$ 16.4 NCAP.mins, respectively (Figure 3.5.C). Moreover, reduced maintenance of the A fibre CAP with 10mM fructose compared to 10mM glucose was further reduced when combined with 4-CIN to 348.6 $\pm$ 62.4 NCAP.mins vs 216.4 $\pm$ 55.8 NCAP.mins, similar to that of no substrate conditions (216.4 $\pm$ 55.8 NCAP.mins vs 151.3 $\pm$ 27.8 NCAP.mins) (Figure 3.5.A and C). Interestingly, when looking at the C fibre CAP 200 μ M 4-CIN did not affect the ability of 10mM glucose or 20mM fructose to maintain the CAP fully for 8 hours; 477.6 $\pm$ 10 NCAP.mins and 488 $\pm$ 10.9 NCAP.mins, respectively (Figure 3.5.B and C). However, in the presence of 10mM fructose the maintenance of the C fibre CAP was significantly reduced compared to 10mM fructose alone (266.3 $\pm$ 12.5 NCAP.mins vs 409.2 $\pm$ 3.2 NCAP.mins, respectively), similar to the A fibre CAP, but was still significantly greater than that under no substrate conditions (266.3 $\pm$ 12.5 NCAP.mins vs 63.5 $\pm$ 4.4 NCAP.mins, respectively), unlike the A fibre CAP (Figure 3.5.B and C).

The changes seen in the CAP maintenance with fructose and 4-CIN led to investigations into the ability of fructose to recover the CAP in the presence of 4-

CIN. Because 10mM fructose alone was unable to recover the A fibre CAP, 10mM fructose with 200 μ M 4-CIN was not used in these experiments. It became immediately apparent that 20mM fructose was now no longer able to recover the A fibre CAP when supplied with 200 μ M 4-CIN (Figure 3.5.D and F), however, again 4-CIN had no significant effect on the ability of 10mM glucose to recover the A fibre CAP, both in terms of the percentage recovery (92.5 $\pm$ 6.9% vs 98.7 $\pm$ 1.9%, respectively) and the time taken to start CAP recovery (8.3 $\pm$ 3.8 mins vs 7.2 $\pm$ 4.4 mins) (Figure 3.5.F and G). In contrast to the A fibre CAP, 4-CIN had no effect on either 10mM glucose or 20mM fructose to recover the C fibre CAP (Figure 3.5.E) in terms of percentage recovery (94.3 $\pm$ 8.3% vs 92.2 $\pm$ 8.8% and 95.1 $\pm$ 7% vs 99.9 $\pm$ 0.1%, respectively) and recovery time (3.0 $\pm$ 1.0 mins vs 1.3 $\pm$ 0.6 mins and 3.5 $\pm$ 0.7 mins vs 1.7 $\pm$ 1.2 mins, respectively) (Figure 3.5.F and G).

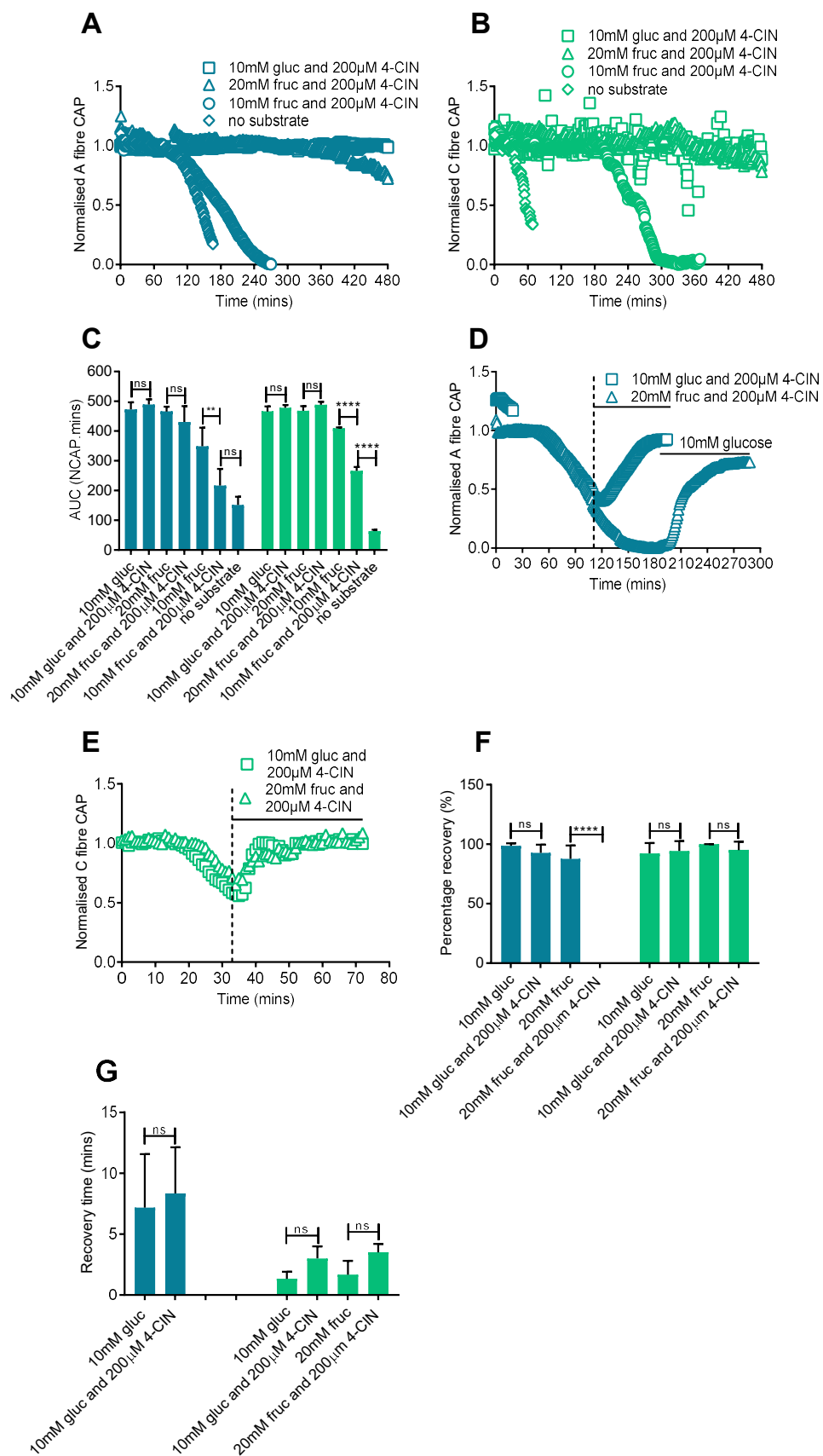


Figure 3.5: 4-CIN affected the ability of fructose to maintain and recover the A but not the C fibre CAP. **(A)** 10mM glucose and 200 μ M 4-CIN fully maintained the A fibre CAP whilst 20mM fructose and 200 μ M 4-CIN resulted in a late fall. 4-CIN with 10mM fructose resulted in maintenance of the A fibre CAP similar to that of no substrate conditions. **(B)** The presence of 200 μ M 4-CIN with either 10mM glucose or 20mM fructose maintained the C fibre CAP for 8 hours. 10mM fructose and 200 μ M 4-CIN maintained the CAP better than no energy substrate. **(C)** 4-CIN had no significant effect on the ability of 10mM glucose or 20mM fructose to maintain the A (n=2 and n=3, respectively) and C (n=3 and n=2, respectively) fibre CAP, but significantly reduced the maintenance of the C (n=3) and A (n=3) fibre, CAP when supplied with 10mM fructose. There was no significant difference in the maintenance of the A fibre CAP, but not the C fibre CAP, between 10mM fructose and 200 μ M 4-CIN, and no substrate conditions. **(D)** The A fibre CAP recovered in the presence of 10mM glucose and 200 μ M 4-CIN, but not with 20mM fructose and 200 μ M 4-CIN. **(E)** The C fibre CAP recovered when 4-CIN was supplied with either 10mM glucose or 20mM fructose. **(F)** 4-CIN had no significant effect on the percentage recovery of the A (n=3) and C (n=3) fibre CAP with 10mM glucose but when supplied with 20mM fructose prevented A fibre CAP (n=3) recovery, but had no significant effect on the C fibre CAP (n=2). **(G)** The time taken for the A fibre CAP to start recovering in 10mM glucose was not significantly effected by 4-CIN. Likewise 4-CIN had no significant effect on C fibre CAP recovery time in 10mM glucose or 20mM fructose. Gluc= glucose and fruc= fructose.

3.6. Lactate release from the sciatic nerve in the presence of glucose and fructose

Due to the finding that blocking lactate transport appeared to reduce the efficiency of fructose as an energy substrate for the A, but not the C fibres, experiments progressed to the use of lactate biosensors in addition to electrophysiology to investigate lactate release from the sciatic nerve in the presence of glucose or fructose, in particular during CAP recovery.

Initially dose-response curves for lactate release from the nerve were determined in the presence of glucose or fructose. Before the nerve was subjected to increasing concentrations of glucose/fructose the lactate electrode was calibrated in 100 μ M lactate in the absence of the sciatic nerve. This enabled the concentration of lactate released from the nerve at the different concentrations of glucose/fructose to be estimated from the lactate signal recorded. When the sciatic nerve was superfused with increasing concentrations of glucose it was found that the lactate released from the nerve also increased up to about 5mM glucose where the lactate signal plateaued ($\sim$ 15 μ M) with further increases in the concentration of glucose (Figure 3.6.A). In contrast when the nerve was exposed to increasing concentrations of fructose (0-30mM) the lactate signal continued to increase (Figure 3.6.B)

The use of lactate biosensors during A fibre CAP recovery experiments were carried out because blocking lactate transport with 4-CIN was particularly striking during CAP recovery. The stable lactate recording during 10mM glucose dropped well before that of the A fibre CAP during no substrate conditions, as described previously by Brown et al (2012) (Figure 3.6.C and D). The addition of 10mM glucose or 20mM fructose resulted in an initial rapid increase in the lactate signal occurring within 1.8 $\pm$ 2.1 mins and 0.3 mins, respectively, from the onset of the energy substrate (Figure 3.6.C, D and F). This initial rise in lactate is described as phase 1 and is followed by a reduced rate of lactate released into the ECS known as phase 2; 0.3 $\pm$ 0.1 N. lactate signal mins⁻¹ vs 0.02 $\pm$ 0.004 Nlactate signal.mins (10mM glucose) and 0.1 Nlactate signal.mins vs 0.03 Nlactate signal.mins (20mM fructose) (Figure 3.6.C, D and E). However,

statistically the rate of the 1st and 2nd phase was not found to be significantly different for 20mM fructose. The beginning of phase 2 coincided with the start of CAP recovery with 10mM glucose (4.2 +/- 1.9 mins vs 7.2 +/- 4.4 mins, respectively) but occurred before the CAP began to recover when supplied with 20mM fructose (4.1 mins vs 35.7 +/- 15.9 mins, respectively) (Figure 3.6.C and D).

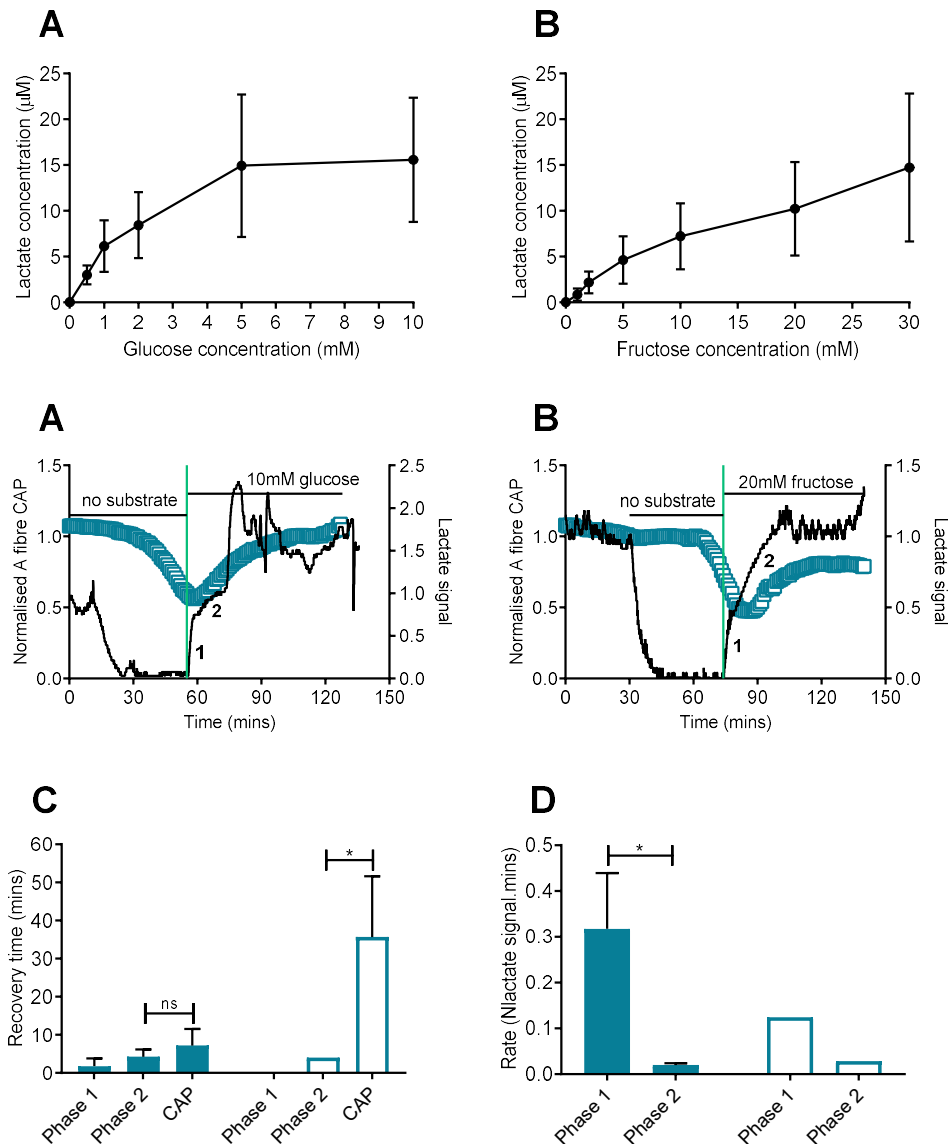
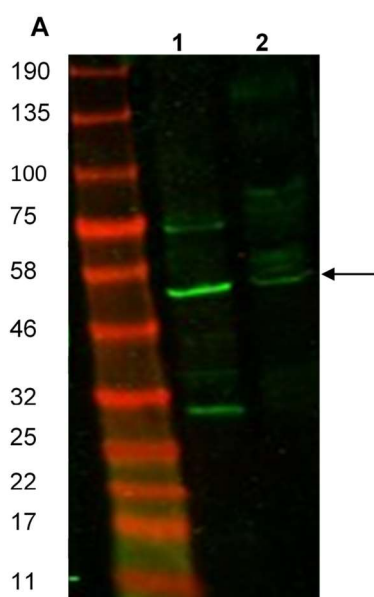


Figure 3.6: Lactate release from the sciatic nerve in the presence of glucose or fructose. **(A)** Glucose-lactate dose-response curve. Lactate released from the sciatic nerve increased up to 5mM glucose where it plateaued ($n=3$). **(B)** Fructose-lactate dose-response curve. As the fructose concentration increased the lactate concentration released from the sciatic nerve increased ($n=7$). **(C)** The lactate signal increased rapidly (1) soon after the supply of 10mM glucose, then increased at a slower rate (2) once the CAP began to recover and plateaued once the CAP was fully recovered. **(D)** The lactate signal increased rapidly (1) soon after the supply of 20mM fructose, then increased at a slower rate (2) once the CAP stopped falling and plateaued when the CAP fully recovered. **(E)** There was no significant difference between the time taken for the CAP to start recovering ($n=6$) and phase 2 of the lactate signal ($n=3$) with 10mM glucose, but there was with 20mM fructose ($n=3$ and $n=1$, respectively). **(F)** The rate of the 1st phase was significantly quicker than that of the 2nd phase during 10mM glucose recovery ($n=3$). Statistical analysis could not be performed on 20mM fructose recovery ($n=1$) but the same result as 10mM glucose would be expected. Solid blue bars= 10mM glucose and empty blue bars= 20mM fructose.

3.7. The expression of fructokinase in the sciatic nerve

Western blotting revealed the expression of fructokinase in the sciatic nerve, although weaker than that in the liver (positive control) (Figure 3.7.A). Fructokinase appeared in both tissues between bands 46 and 58kDa but also appeared at about 32kDa and a fainter band at 75kDa in the liver (Figure 3.7.A). One reason for the appearance of multiple bands for the same protein is alternative splicing of a gene resulting in different forms of the same enzyme (Abcam 2017). This broad finding of fructokinase expression led to further investigation into its location within the nerve. From the negative controls performed it is clear that the staining seen for fructokinase and neurofilament in both sciatic nerve sections and the liver were specific thus conclusions could be made (Figure 3.7.B, C and D inserts). The slightly brighter staining seen in the no primary controls of the longitudinal sciatic nerve sections is likely to be the result of an edge effect rather than non-specific binding (Figure 3.7.D inserts).

Fructokinase was co-stained alongside neurofilament to determine whether it was expressed by the axons. This staining clearly revealed a co-localisation with all axons of the sciatic nerve when looked at in both cross and longitudinal sections (Figure 3.7.C and D). Due to the apparent absence of fructokinase staining where neurofilament staining was not present, it is implied that the Schwann cells do not express fructokinase (Figure 3.7.C and D).



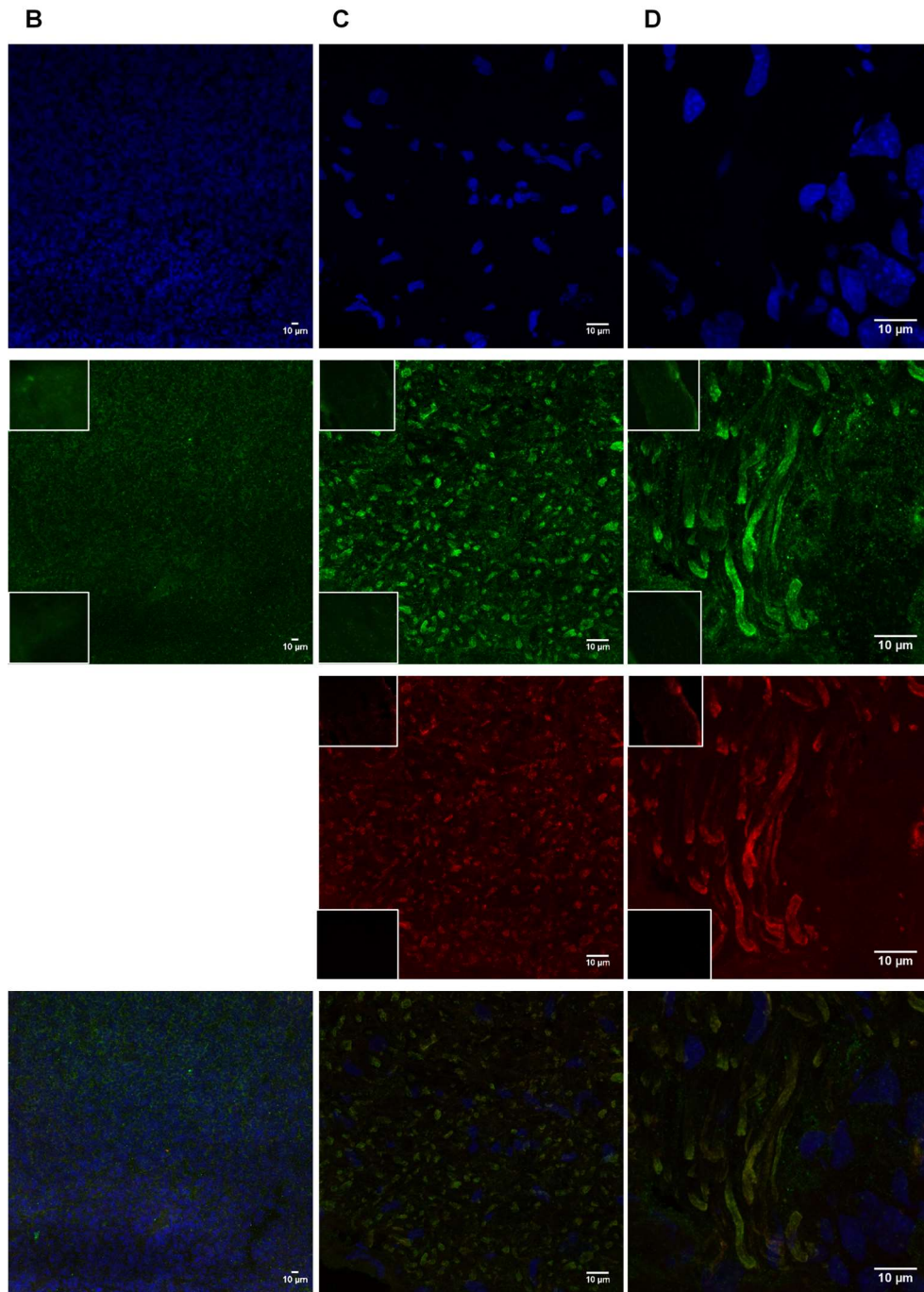


Figure 3.7: Fructokinase expression in the sciatic nerve. **(A)** Western blot of fructokinase in the liver (**1**) and sciatic nerve (**2**), present in both between 46 and 58kDa (arrow). **(B)** Immunohistochemical staining of liver with fructokinase antibody (green) and DAPI (blue) showed abundant expression in hepatocytes. **(C)** Immunohistochemical staining of cross sciatic nerve section with fructokinase antibody (green), neurofilament antibody (red) and DAPI (blue) showed co-localisation of fructokinase with neurofilament. **(D)** Immunohistochemical staining of longitudinal sciatic nerve section with fructokinase antibody (green), neurofilament antibody (red) and DAPI (blue) showed co-localisation of fructokinase with neurofilament. Inserts of controls without primary (top left) and without secondary (bottom left) antibody (x20).

Chapter 4: Discussion

The data from my MRes has built upon established knowledge and added further complexity to the metabolic interactions between Schwann cells and axons of the sciatic nerve particularly in terms of fructose metabolism.

First of all, the significantly better maintenance of the A fibre CAP compared to that of the C fibre CAP in the absence of an exogenous energy substrate was confirmed. Metabolic properties of the sciatic nerve were then further investigated and it was revealed that both A and C fibre conduction failed rapidly in the presence of 1mM cyanide and 10mM glucose, whilst the C fibre CAP was better maintained than the A fibre during 1mM iodoacetate and 20mM lactate compared to their respective maintenance during no energy substrate.

With regards to fructose metabolism it was shown that C fibre conduction can be maintained and recovered using lower concentrations of fructose than the A fibres and that a supply of lactate was required by the A fibres, but not the C fibres, during fructose metabolism. Finally, fructokinase appeared to be expressed by all axons of the sciatic nerve but unlikely to be present in Schwann cells.

4.1. Schwann cell glycogen-derived lactate supports A fibre, but not C fibre, conduction during removal of an exogenous energy substrate

The significantly greater maintenance of A fibre conduction compared to that of the C fibres in the presence of no energy substrate confirms the previous finding by Brown et al (2012) that the A fibre CAP has a longer latency to failure than the C fibre CAP during aglycemia. This differential response between the fibre types to aglycemia was concluded to be the result of glycogen, which is located in the myelinating Schwann cells that myelinate the A fibres but not in the non-myelinating Schwann cells that enclose the C fibres into Remak bundles (Brown et al 2012). Glycogen is metabolised ultimately to lactate which is then supplied by the Schwann cells to the A fibre axons, but not the C fibre axons, providing a source of energy during times of aglycemia (Brown et al 2012) i.e. no substrate conditions (Figure 4.1).

Despite C fibres being unable to benefit from the Schwann cell glycogen-derived lactate their conduction is maintained for longer than that of the axons of the mouse optic nerve (MON). This is likely to be the result of the lower metabolic rate of the sciatic nerve (Dr Paul Smith, personal communication) compared to optic nerve, enabling axons to slowly use up metabolites available during energy substrate deprivation, rather than the efficiency of glucose as an energy substrate. In addition, this experiment revealed another difference between the CNS MON model and the PNS sciatic nerve model of glial-axonal energy metabolism. In the CNS it appears that all axons can benefit from astrocytic glycogen-derived lactate (Brown et al 2003) whereas this does not seem to be the case in the PNS in that only certain subpopulations of axons can utilise lactate provided by Schwann cells. This may be explained by the close contact of the myelinating Schwann cells with the A fibres but not the C fibres, but could also be the result of the large ECS of the sciatic nerve compared to that of the CNS (Hoppe et al 1991).

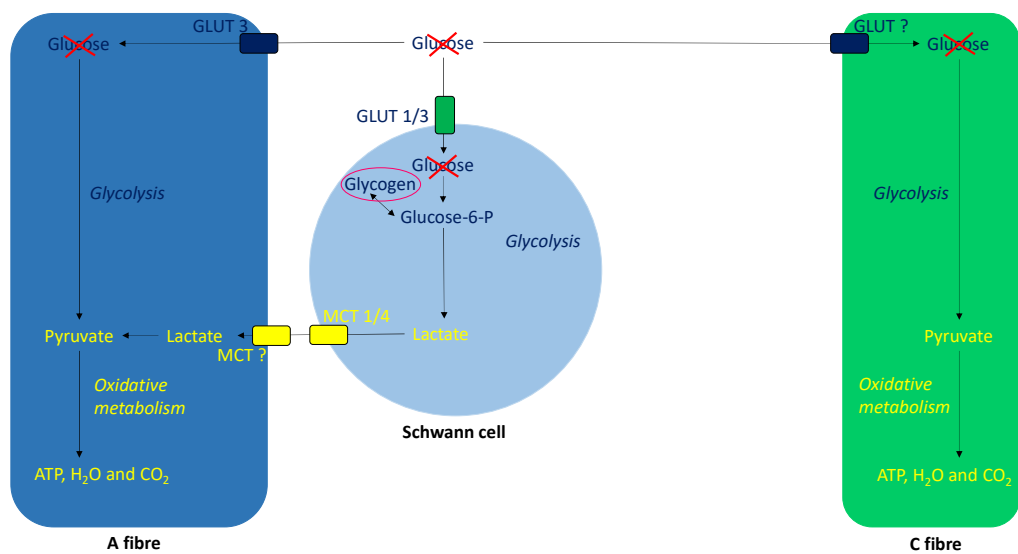


Figure 4.1: Removal of an exogenous energy substrate to the sciatic nerve. All cell types of the sciatic nerve can utilise glucose, but when the glucose supply is compromised (red crosses) only the A fibres benefit from Schwann cell glycogen-derived lactate (circled). It is also likely that the Schwann cells utilise glycogen under these conditions, however these experiments do not attempt to address this.

4.2. Differential requirement for glycolytic and oxidative metabolism between fibre types and Schwann cells

The use of metabolic inhibitors that block distinct metabolic pathways, shed light into the differences in requirement of glycolysis and oxidative metabolism both between the fibre subpopulations and Schwann cells.

Superfusion of the sciatic nerve with 10mM glucose and 1mM iodoacetate, a glycolytic inhibitor, significantly reduced the maintenance of both the A and C fibre CAP below that of their respective maintenance of conduction during no energy substrate. This is explained by the site of action of iodoacetate along the glycolytic pathway. Iodoacetate inhibits the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sabri & Ochs 1971) which occurs about half way down the pathway (6th enzyme out of 10) (Champe et al 2008), this means the axons were no longer able to use the supplied glucose as this enters the pathway above GAPDH, but instead used up the remaining metabolites below that of GAPDH available at the addition of iodoacetate (Figure 4.2). Furthermore, although statistically significant the maintenance of the A fibre CAP during 10mM glucose and 1mM iodoacetate could be considered as similar to that of the C fibre CAP under the same conditions. This may initially not be what would be expected considering A fibre conduction is better maintained under no substrate conditions than the C fibres. However, iodoacetate blocks glycolysis not only in the axons but also in the Schwann cells thus the glucose-6-phosphate from glycogen breakdown would no longer be metabolised to lactate for A fibre benefit i.e. akin to the C fibres during no energy substrate (Figure 4.2.). Therefore the similarity between A and C fibre CAP maintenance in the presence of 10mM glucose and 1mM iodoacetate provided additional evidence that A fibres benefit from Schwann cell glycogen-derived lactate but not the C fibres.

Inhibition of complex IV of the electron transport chain, by cyanide (Champe et al 2008), also resulted in a significant reduction in maintenance of the A and C fibre conduction compared to no substrate conditions, even when in the presence of 10mM glucose. This clearly implies that neither A or C fibres can maintain conduction on glycolysis alone, due to the lack of benefit of the glucose

that is still glycolytically metabolised when cyanide is present (Figure 4.2). This response of both the A and C fibres is in contrast to that of the optic nerve in which a large proportion of axons continued to conduct in the presence of cyanide (Tekkok et al 2003).

A particularly interesting aspect to these experiments was the use of iodoacetate with lactate as the exogenously supplied energy substrate. Lactate was supplied at a concentration of 20mM as this is the carbon equivalent of the standard 10mM glucose. It has already been shown by Brown et al (2012) that both the A and C fibre CAP are maintained in the presence of 20mM lactate, and because of the understanding that the A fibres benefit from glycogen-derived lactate, it would be plausible to hypothesise that A fibre conduction would be fully maintained in 20mM lactate even with the addition of iodoacetate, as lactate would bypass the glycolytic pathway and enter oxidative metabolism (Figure 4.2). However this was not found to be the case, but instead the A and C fibre CAP were similarly maintained to that of the A fibre CAP under no substrate conditions i.e. 20mM lactate and 1mM iodoacetate provided no additional benefit to the A fibres, but did for the C fibres, compared to the absence of any exogenously supplied energy substrate. In terms of the A fibres, this suggests that they can use lactate as an energy substrate to maintain conduction to some extent but also require glycolysis for full maintenance of conduction (Figure 4.2). The response of the C fibres to this condition adds another layer of interest. Despite being unable to benefit from glycogen-derived lactate, Brown et al (2012) showed their ability to maintain the CAP using lactate, thus it may also be hypothesised that the C fibres would conduct fully in 20mM lactate and 1mM iodoacetate (Figure 4.2). Once again this was not the case, but instead of a similar maintenance of conduction to removal of an energy substrate, the C fibre CAP showed significantly increased maintenance suggesting they are able to survive on oxidative metabolism alone (Figure 4.2). Why the C fibres failed to fully maintain conduction under these conditions is therefore intriguing but one possible explanation is that the Schwann cells cannot function solely on oxidative metabolism and possibly depend more on glycolytic metabolism; akin to

astrocytes (Bouzier-Sore et al 2006). This preference for glycolysis by the Schwann cells explains the maintenance of conduction seen with 20mM lactate as during this the Schwann cells would produce energy from glycolytic metabolism of glycogen. This is supported by the finding from Viader et al (2011) that mitochondrial dysfunction specific to Schwann cells of the mouse sciatic nerve had no impact on Schwann cell survival up to the 8 months of age, suggesting that Schwann cells of, at least, young sciatic nerve can survive on the proceeds of glycolysis which is further strengthened by Vega et al (2003) who showed that Schwann cells take up about 75% of the available glucose. Therefore under these conditions the Schwann cells would eventually die and release their contents, including K^+ into the ECS. Extracellular K^+ is usually buffered to a large extent by the Schwann cells and a smaller extent by the axons (Kofuji & Newman 2004). However, now the Schwann cells would be unavailable to buffer the K^+ and the C fibres unable to buffer it sufficiently due to the possible requirement of glycolytic ATP (blocked by iodoacetate) by the Na^+/K^+ ATPase (Okamoto et al 2001, Sepp et al 2014) or as a result of the large ECS (Hoppe et al 1991), thus resulting in constant depolarisation of the C fibres and eventually death. This is an unavoidable consequence of the mixed nature of sciatic nerve.

Altogether it can be implied that A fibres require both glycolytic and oxidative metabolism, while C fibres can survive solely on oxidative metabolism, and we presume that Schwann cells rely on glycolytic metabolism.

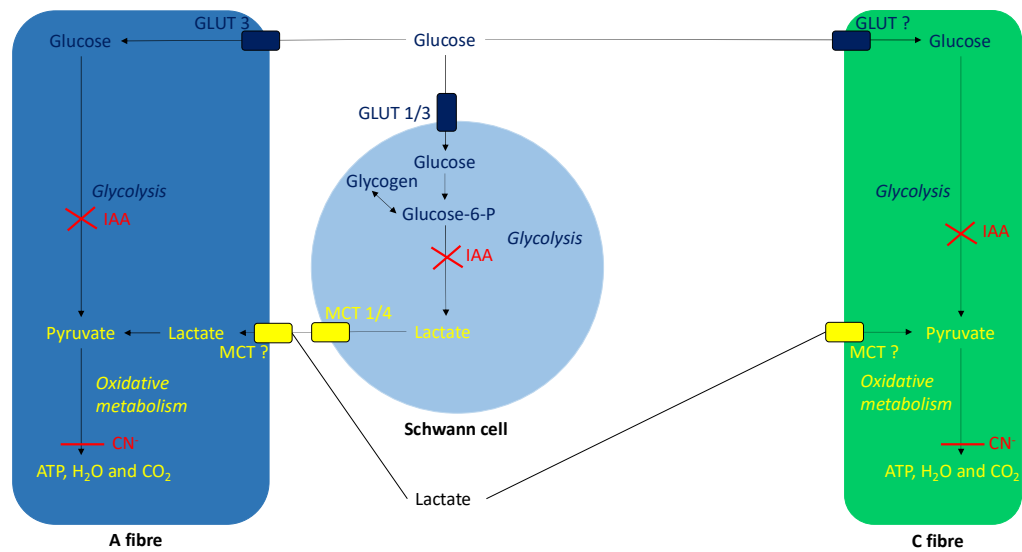


Figure 4.2: Metabolic inhibitors in the sciatic nerve. Iodoacetate (red crosses) blocks mid-way along the glycolytic pathway preventing the use of glucose by all cell types, and glycogen, by the A fibres and Schwann cells. Lactate can bypass the effect of iodoacetate and directly enter oxidative metabolism, supporting the C fibres and, to some extent, the A fibres but not the Schwann cells. Despite cyanide (red line) acting at the end of oxidative metabolism ATP from the glycolytic metabolism of glucose is not sufficient to support A and C fibre conduction in the presence of cyanide. IAA= iodoacetate and CN⁻= cyanide.

4.3. A fibres require a higher concentration of fructose than C fibres

10mM and 20mM were chosen as the two main concentrations of fructose as these were the concentrations used by Allen et al (2006) and Meakin et al (2007) to study fructose metabolism of the optic nerve, allowing comparisons between models.

It appears that the C fibre CAP is better maintained than the A fibre CAP in the different concentrations of fructose. Despite the significant differences found between 10mM and 5mM fructose to 10mM glucose C fibre CAP maintenance, this is likely to be the result of the small standard deviations for the two fructose concentrations thus may not represent a true significant difference. This is clear from the CAP traces and particularly the significant difference between C fibre CAP maintenance in 5mM fructose and no substrate conditions, which was found not to be significant in terms of the A fibre CAP. Furthermore, the decline in the C fibre CAP maintenance at lower concentrations of fructose could again be due to a large increase in extracellular K^+ as result of A fibre death, which may not be buffered sufficiently causing C fibre failure.

To further establish whether A fibres require higher concentrations of fructose than C fibres, CAP recovery using fructose was investigated. Recovery of the CAP requires more energy than simply maintaining the CAP due to the substantial energy required to restore ionic gradients, by the Na^+/K^+ ATPase, and repolarise all membranes. Therefore these experiments can be seen as providing greater insight into the efficiency of fructose as an energy substrate for each fibre type compared to the maintenance experiments. In addition, when the sciatic nerve is subjected to energy substrate deprivation, the glycogen present in the Schwann cells is broken down and the lactate used up by the A fibres, thus when fructose is supplied there is no glycogen present and any recovery in the A fibre CAP seen is the result of fructose metabolism not glycogen. The requirement for higher concentrations of fructose by the A fibres compared to that of the C fibres was evident from the recovery experiments in that 20mM fructose could recover both CAPs but 10mM fructose could only recover the C fibre CAP not only completely but also as quickly as 10mM glucose and 20mM fructose. An

interesting observation of these recovery experiments was the long length of time after the addition of fructose and the start of A fibre CAP recovery compared to that of when 10mM glucose was supplied, despite a fairly comparable percentage recovery. This long latency to recovery was also observed in the MON (Meakin et al 2007) and hippocampus (Izumi & Zorumski 2009).

The recovery in the A fibre CAP seen with 20mM fructose implies it is the result of fructose metabolism rather than glycogen-derived lactate. This ability of the CAP to be maintained by fructose metabolism was also investigated by preincubating the nerve in 10mM glucose, 20mM fructose or 10mM fructose, for sufficient time but before the CAP began to fall, then removing an exogenous supply of energy substrate. The similarity in maintenance of the A fibre CAP between preincubation in 10mM glucose and 20mM fructose suggests that glycogen is either not being broken down or its level is maintained under these conditions. While the CAP maintenance is significantly reduced when preincubated in 10mM fructose to a similar maintenance of the C fibre CAP when subjected to no substrate, suggesting glycogen is being broken down when the nerve is superfused with 10mM fructose. Therefore A fibre conduction is at least to some extent maintained by Schwann cell glycogen-derived lactate when supplied with 10mM fructose.

From this set of experiments it was concluded that A fibres require a higher concentration of fructose to maintain conduction compared to that of the C fibres. This difference in axon type requirement of fructose concentration is similar to that seen in the MON between different sized axons. Meakin et al (2007) concluded that smaller diameter axons could effectively use lower concentrations of fructose because they expressed fructokinase (lower K_m and higher V_{max} for fructose than hexokinase) whereas the larger diameter axons did not, thus in these axons fructose is metabolised by hexokinase, a high K_m and low V_{max} reaction hence the requirement for a higher concentration of fructose. From this it could be hypothesised that the higher concentration requiring A fibres do not express fructokinase but the lower concentration

requiring C fibres do (further discussed in 4.5). Moreover, the significantly long time before A fibre CAP recovery in the presence of 20mM fructose compared to 10mM glucose led to the hypothesis of a role of Schwann cell fructose-derived lactate, based on Izumi and Zorumski (2009) who showed that neurones of the hippocampus indirectly metabolise fructose via glial cell supply of fructose-derived monocarboxylates.

4.4. Possible role of Schwann cell fructose-derived lactate in supporting A fibre conduction

The involvement of lactate in the ability of fructose to support A fibre conduction was first investigated using 4-CIN, a blocker of MCT2 thus lactate transport (Wender et al 2000). It is important to note that despite the little available evidence for the precise expression of MCT2 by the sciatic nerve it has been found to be present (Domenech-Estevéz et al 2015) and since it is the Schwann cells not the axons that release lactate, and the effect observed by 4-CIN on the maintenance of the A fibre CAP even when no exogenous lactate was supplied, we conclude that the Schwann cells metabolise fructose to lactate and this is transferred to the axons.

First of all 4-CIN was supplied to the nerve alongside 10mM glucose, providing insight into whether A fibre axons use glycogen-derived lactate under normoglycemic conditions as well as aglycemic/no substrate conditions. It is clear that the presence of 4-CIN had no effect on the ability of 10mM glucose to fully maintain either the A or C fibre CAP, confirming the finding by Vega et al (2003) using the vagus nerve (a peripheral nerve), suggesting either the A fibres do not use glycogen-derived lactate when glucose is in plentiful supply, or that the A fibres are able to compensate for the inhibition of lactate transport by using more glucose. Moreover, the absence of effect of 4-CIN on CAP maintenance in the presence of 10mM glucose is akin to the optic nerve under the same conditions (Wender et al 2000).

It is clear from the CAP traces that 4-CIN had more of an effect on the maintenance of the A fibre than C fibre conduction, suggesting a requirement for Schwann cell fructose-derived lactate by the A fibres. Furthermore, the significant reduction in maintenance of the C fibre CAP in the presence of 10mM fructose and 200 μ M 4-CIN compared to 10mM fructose alone is likely to be the result of the reduced maintenance of the A fibre CAP to that of during no substrate, again resulting in an increase in extracellular K⁺. This effect can be seen as providing additional support for the reduced maintenance of A fibre conduction as a result of blocked lactate transport when supplied with fructose,

particularly as 10mM fructose and 200 μ M 4-CIN is statistically equivalent to no substrate conditions.

Once again CAP recovery experiments were performed and from these a more obvious role of lactate in maintaining A fibre conduction, but not C fibre, in the presence of fructose was observed. The involvement of lactate in CAP recovery when supplied with 10mM glucose was first established, and it was found that blocking lactate transport had no effect on either the amount the CAP recovers to, or the time taken to start recovering for both the A and C fibres. This again suggests that either the lactate released by Schwann cells is not used by the A fibres during CAP recovery or that they can compensate with glucose uptake when this lactate is unavailable. However, the effect of 4-CIN on A fibre CAP recovery was evident when supplied with 20mM fructose; the slow but substantial recovery in the A fibre CAP previously seen with 20mM fructose was now completely abolished clearly indicating a requirement for lactate by the A fibres from the Schwann cells in the presence of fructose. In contrast, no significant effect of 4-CIN was seen on the ability of the C fibres to recover conduction in terms of both percentage recovery and the time taken, suggesting, as expected, C fibres do not require lactate from the Schwann cells when supplied with fructose .

The small effect of 4-CIN on C fibre conduction clearly suggests that they do not take up lactate from the Schwann cells in the presence of fructose, thus it is likely they metabolise fructose directly. In contrast, from these recovery and maintenance experiments it could be concluded that the A fibres have an absolute requirement for Schwann cell lactate in the presence of fructose, however in the presence of 20mM fructose and 200 μ M 4-CIN the maintenance of the A fibre CAP is greater than that during no substrate conditions implying that the A fibres must also take up fructose themselves in addition to the Schwann cells. Therefore, it is likely that the A fibres take up fructose and metabolise directly, akin to the C fibres, to some extent but particularly when in more energy requiring conditions, i.e. CAP recovery, Schwann cell supplied lactate is also required. This is supported by the expression of GLUT5 (the

fructose transporter) on both the axons and Schwann cells (Asada et al 1998). A fibre fructose metabolism is, to some extent, comparable to that of neurones in the CNS hippocampus (Izumi & Zorumski 2009) in that they require fructose to be metabolised by the glial cell to a monocarboxylate but differ by also metabolising fructose themselves.

Lactate biosensors were then incorporated into the electrophysiology experiments in order to further investigate the role of lactate in maintaining A fibre conduction in the presence of fructose. From the initial glucose-lactate and fructose-lactate dose-response curves it was clear that lactate is produced within the sciatic nerve when supplied with either glucose or fructose, confirming its production by the Schwann cells.

During CAP recovery experiments, when the nerve was subjected to no substrate conditions the lactate signal dropped soon after the onset as the result of the A fibres rapidly taking up any lactate released by the Schwann cells from glycogen breakdown, thus little remains in the ECS (Brown et al 2012). An initial observation of these experiments is the two phases of lactate signal recorded with both 10mM glucose and 20mM fructose. The initial, rapid increase in lactate signal in response to the addition of the energy substrate implies a rapid uptake and metabolism of the energy substrate by the Schwann cells. The second, slower rising phase suggests either the Schwann cells release less lactate or that more of the lactate being released by the Schwann cells is taken up by the axons and oxidatively metabolised. If the latter is true then during CAP recovery in 10mM glucose, the A fibres do take up Schwann cell released lactate when it is available but can compensate when this transport is blocked, whilst recovery in 20mM fructose requires the uptake of lactate. However, it is important to note that the biosensors used only record the lactate that escapes into the surrounding solution of the bath thus it does not mean that no lactate is taken up by the axons in the 1st phase but that it is also released into the ECS.

An intriguing aspect of the lactate electrode recordings is the time difference between the 2nd phase and the start of CAP recovery with 10mM glucose and 20mM fructose. When supplied with 10mM glucose the A fibre CAP started to

recover at a similar time to that of the onset of the 2nd phase of the lactate signal, this is likely to be because the A fibres can recovery solely on 10mM glucose without lactate as this provides sufficient energy from both glycolysis and oxidative metabolism. However, due to recording lactate in the ECS during 20mM fructose recovery it appeared that a requirement for Schwann cell fructose-derived lactate in order to recover the A fibre CAP in 20mM fructose may not be the full explanation for why the CAP takes so long to the recover. This led to investigations into the expression of fructokinase in different cellular compartments of the sciatic nerve.

4.5. Fructokinase expression in the sciatic nerve

From the initial Western blot fructokinase appeared to be expressed within the sciatic nerve. The pattern of expression of fructokinase in the liver is similar to that found in the liver by Diggle et al (2009), using an N-terminal antibody for fructokinase, and may indicate that the band present in both tissues represents isoform C, not A, of fructokinase. This may further explain why fructokinase in the sciatic nerve is present at a band different to that of the optic nerve found at 38kDa (Meakin et al 2007).

Immunohistochemical analysis for the cellular location of fructokinase suggests it is expressed by all axons but unlikely to be expressed by the Schwann cells; contradictory to what was expected. The expression of fructokinase by the C fibres, however, was expected and explains the ability of C fibres to maintain and recover conduction in the presence of lower fructose concentrations (Figure 4.5). As the A fibres also express fructokinase it may be expected that they can survive on lower concentrations of fructose, however this was established not to be the case, and that in the presence of fructose they also require lactate released by Schwann cells (Figure 4.5). Schwann cells on the other hand do not appear to express fructokinase, and thus it is implied that they metabolise fructose via hexokinase, an initial glycolytic enzyme that has a much higher K_m for fructose than fructokinase hence why A fibre conduction is maintained and recovered only in higher concentrations of fructose (Figure 4.5). Therefore, a possible explanation for why the A fibre CAP takes so long to start to recover in 20mM fructose is that the Schwann cells require such a large amount of fructose in order to metabolise it that very little may initially be available to the A fibres and even though sufficient lactate may be available, as revealed by the use of metabolic inhibitors, A fibres show a preference for energy from both glycolytic and oxidative metabolism (both of which are provided with glucose, hence the rapid recovery).

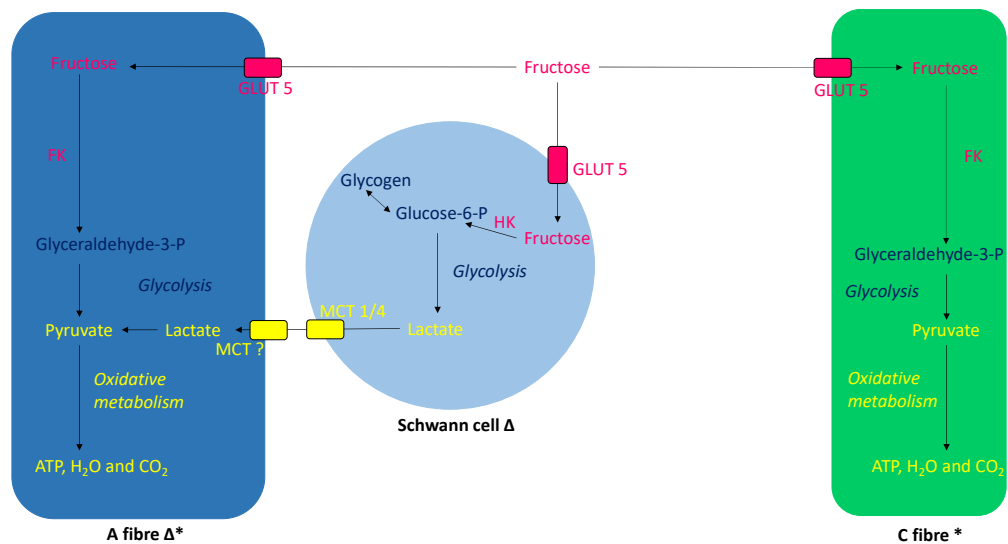


Figure 4.5: Fructose metabolism in the sciatic nerve. C fibres metabolise fructose directly via fructokinase even at lower concentrations. The A fibres require Schwann cell fructose-derived lactate, metabolised via hexokinase in the Schwann cells, but also metabolise fructose directly via fructokinase due to a preference for both glycolytic and oxidative metabolism therefore need higher concentrations in order for the Schwann cells to meet their energy demands preferentially by glycolysis. Δ = glycolytic and $*$ = oxidative metabolism.

4.6. Future directions

Future directions include those related to this study and those on a much broader scale. In addition to repeats, co-staining for Schwann cells (S100B stain) and fructokinase is required to confirm whether or not they express fructokinase. An inhibitor of glycogen breakdown e.g. DAB could also be used to confirm the role of glycogen metabolism during CAP maintenance in the presence of fructose. As well as the MCT inhibitor (4-CIN) an inhibitor of GLUT transporters e.g. CCB could also be used to investigate fructose uptake via GLUT transporters. This would be particularly interesting as CCB is the inhibitor used by Izumi and Zorumski (2009), but blocks GLUT1-4 not 5. Alongside this, staining for GLUT and MCTs could be performed to confirm their presence on the different cellular components of the sciatic nerve and provide further insight into the sites of uptake of fructose. Furthermore, repeats of experiments already performed using more concentrations of fructose would be interesting. It would be likely that increasing the concentration above 20mM would have no additional benefit to axon conduction but using concentrations between 10mM and 20mM could reveal the lowest concentration of fructose required to be as effective as 20mM fructose/10mM glucose. Finally ion sensitive electrodes, particularly for K^+ , could be combined with electrophysiology to confirm the increased extracellular K^+ that may prevent C fibre conduction in lower fructose concentrations and investigate whether K^+ is the signal that stimulates Schwann cell lactate release for use by the A fibres, as occurs between axons and astrocytes in the CNS (Choi et al 2012).

An area that further studies could progress towards is diabetes, in particular as the sciatic nerve is a well-known target of diabetic neuropathy. The concentration of blood glucose that occurs during diabetes is thought to be toxic to the axons resulting in impaired function and degeneration of C fibres and A β fibres (Lennertz et al 2011). From this study and the study by Brown et al (2012) it is clear that A fibres benefit from glycogen stored in the myelinating Schwann cells not the non-myelinating Schwann cells around the C fibres. It may be possible that the C fibres are affected during diabetes due to the lack of

protective barrier of the myelinating Schwann cells to store the excess glucose as glycogen. Experiments to consider could include superfusing the sciatic nerve with very high concentrations of glucose e.g. 50mM and determining any effect on the A and C fibre CAP compared to control, 10mM glucose. Additionally, comparisons between the maintenance of conduction of A fibres in particular between normal and diabetic nerves when subjected to no substrate conditions may provide an indication of the role of the Schwann cell store of glycogen during diabetes.

4.7. Limitations

The most prevalent limitation of this study is the lower n numbers. This was partly due to the time constraint of completing the MRes but also due to the difficult recording technique. In particular the electrophysiology which required substantially practice and some optimisation initially.

Another aspect of the study that could be improved is the use of null biosensors. These could be used alongside the lactate biosensors and used as a control to cancel out any non-specific interferants that could also be detected by the lactate electrodes (Karagiannis et al 2016).

Chapter 5: Conclusions

In conclusion, these experiments have confirmed the previous finding by Brown et al (2012) that the A fibres are better maintained than C fibres during times of aglycemia/no substrate due to benefiting from Schwann cell glycogen-derived lactate. This was then further validated by exposing the sciatic nerve to 10mM glucose and 1mM iodoacetate as the A fibres were no longer able to benefit from Schwann cell glycogen resulting in attenuated maintenance of conduction similar to that of the C fibre. This finding makes it clear that the universal theory of metabolic interactions between axons and glial cells applies to astrocytes and axons of the CNS, and Schwann cells and A fibres of the PNS but not C fibres.

The use of metabolic inhibitors also uncovered differences in requirement for glycolytic and oxidative metabolism between the different cellular compartments of the sciatic nerve. The A fibres appear to require both glycolytic and oxidative metabolism, while the C fibre can survive solely on oxidative metabolism and the Schwann cells on glycolytic metabolism.

Investigations into fructose as a sufficient energy substrate for the sciatic nerve also revealed cooperation between Schwann cells and A fibres but independent function of the C fibres. The C fibres appear to maintain and recover conduction at much lower concentrations of fructose equally as well as normoglycemic conditions as the result of expressing the high fructose affinity enzyme, fructokinase, thus take up and metabolise fructose directly. The A fibres, on the other hand, conduct in the presence of higher concentrations of fructose, due to metabolising fructose directly, via fructokinase, as well as a requirement for fructose-derived lactate released by Schwann cells that do not appear to express fructokinase, but instead metabolise fructose via hexokinase, a low fructose affinity enzyme. Altogether this data provides an insight into fructose metabolism and the associated metabolic glia-axon interactions of the sciatic nerve from a non-diabetic source, thus may be used to help understand changes seen under diabetic conditions.

Abbreviations

4-CIN	α -Cyano-4-hydroxycinnamic acid
ACh	Acetylcholine
aCSF	Artificial cerebrospinal fluid
AUC	Area under the curve
BBB	Blood brain barrier
BNB	Blood nerve barrier
BSA	Bovine serum albumin
CAP	Compound action potential
CCB	Cytochalasin B
CNS	Central nervous system
ECF	Extracellular fluid
ECS	Extracellular space
ETC	Electron transport chain
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT	Glucose transporter
H ⁺	Proton
HCO ₃ ⁻	Bicarbonate
HFCS	High-fructose corn syrup
HFS	High frequency stimulation
LDH	Lactate dehydrogenase
MCT	Monocarboxylate transporter
MON	Mouse optic nerve
NBC	Sodium-bicarbonate cotransporter
NCAP.mins	Normalised CAP x minutes
NH ₄ ⁺	Ammonium
Nlactate signal.mins	Normalised lactate signal x minutes
PBS	Phosphate buffered saline
PDC	Pyruvate dehydrogenase complex
PS	Population spike
RON	Rat optic nerve
sAC	Soluble adenylyl cyclase
TCA	Tricarboxylic acid cycle

References

- Abcam. 2017. Western blot troubleshooting tips.
- Abrams CK, Rash JE. 2009. Connexins in the Nervous System In *Connexins: A guide*. New York: Humana Press
- Allen L, Anderson S, Wender R, Meakin P, Ransom BR, et al. 2006. Fructose supports energy metabolism of some, but not all, axons in adult mouse optic nerve. *J Neurophysiol* 95: 1917-25
- Allt G, Lawrenson JG. 2000. The blood-nerve barrier: Enzymes, transporters and receptors - A comparison with the blood-brain barrier. *Brain Res Bull* 52: 1-12
- Amaral AI. 2013. Effects of hypoglycaemia on neuronal metabolism in the adult brain: role of alternative substrates to glucose. *J Inherit Metab Dis* 36: 621-34
- Asada T, Takakura S, Ogawa T, Iwai M, Kobayashi M. 1998. Overexpression of glucose transporter protein 5 in sciatic nerve of streptozotocin-induced diabetic rats. *Neurosci Lett* 252: 111-4
- Attwell D, Laughlin SB. 2001. An energy budget for signaling in the grey matter of the brain. *J Cerebr Blood F Met* 21: 1133-45
- Barros LF. 2013. Metabolic signaling by lactate in the brain. *Trends Neurosci* 36: 396-404
- Barros LF, Deitmer JW. 2010. Glucose and lactate supply to the synapse. *Brain Res Rev* 63: 149-59
- Barros LF, San Martin A, Sotelo-Hitschfeld T, Lerchundi R, Fernandez-Moncada I, et al. 2013. Small is fast: astrocytic glucose and lactate metabolism at cellular resolution. *Front Cell Neurosci* 7: 27
- Bear MF, Connors BW, Paradiso MA. 2007. *Neuroscience: Exploring the Brain*. pp. 51-74, 75-100 and 23-50. London: Lippincott Williams and Wilkins.
- Becker HM, Broer S, Deitmer JW. 2004. Facilitated lactate transport by MCT1 when coexpressed with the sodium bicarbonate cotransporter (NBC) in *Xenopus* oocytes. *Biophys J* 86: 235-47
- Belanger M, Allaman I, Magistretti PJ. 2011. Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation. *Cell Metab* 14: 724-38
- Benington JH, Heller HC. 1995. Restoration of Brain Energy-Metabolism as the Function of Sleep. *Prog Neurobiol* 45: 347-60
- Bergersen LH. 2007. Is lactate food for neurons? Comparison of monocarboxylate transporter subtypes in brain and muscle. *Neuroscience* 145: 11-9
- Bittar PG, Charnay Y, Pellerin L, Bouras C, Magistretti PJ. 1996. Selective distribution of lactate dehydrogenase isoenzymes in neurons and astrocytes of human brain. *J Cereb Blood Flow Metab* 16: 1079-89
- Bouzier-Sore AK, Merle M, Magistretti PJ, Pellerin L. 2002. Feeding active neurons: (re)emergence of a nursing role for astrocytes. *J Physiol Paris* 96: 273-82
- Bouzier-Sore AK, Pellerin L. 2013. Unraveling the complex metabolic nature of astrocytes. *Front Cell Neurosci* 7: 179
- Bouzier-Sore AK, Voisin P, Bouchaud V, Bezancon E, Franconi JM, Pellerin L. 2006. Competition between glucose and lactate as oxidative energy substrates in both neurons and astrocytes: a comparative NMR study. *Eur J Neurosci* 24: 1687-94
- Broer S, Broer A, Schneider HP, Stegen C, Halestrap AP, Deitmer JW. 1999. Characterization of the high-affinity monocarboxylate transporter MCT2 in *Xenopus laevis* oocytes. *Biochem J* 341: 529-35
- Broer S, Rahman B, Pellegrini G, Pellerin L, Martin JL, et al. 1997. Comparison of lactate transport in astroglial cells and monocarboxylate transporter 1 (MCT 1) expressing *Xenopus laevis* oocytes. Expression of two different

- monocarboxylate transporters in astroglial cells and neurons. *J Biol Chem* 272: 30096-102
- Broer S, Schneider HP, Broer A, Rahman B, Hamprecht B, Deitmer JW. 1998. Characterization of the monocarboxylate transporter 1 expressed in *Xenopus laevis* oocytes by changes in cytosolic pH. *Biochem J* 333: 167-74
- Brown AM, Baltan Tekkok S, Ransom BR. 2004. Energy transfer from astrocytes to axons: the role of CNS glycogen. *Neurochem Int* 45: 529-36
- Brown AM, Evans RD, Black J, Ransom BR. 2012. Schwann cell glycogen selectively supports myelinated axon function. *Ann Neurol* 72: 406-18
- Brown AM, Sickmann HM, Fosgerau K, Lund TM, Schousboe A, et al. 2005. Astrocyte glycogen metabolism is required for neural activity during aglycemia or intense stimulation in mouse white matter. *J Neurosci Res* 79: 74-80
- Brown AM, Tekkok SB, Ransom BR. 2003. Glycogen regulation and functional role in mouse white matter. *J Physiol* 549: 501-12
- Burant CF, Takeda J, Brotlaroché E, Bell GI, Davidson NO. 1992. Fructose Transporter in Human Spermatozoa and Small-Intestine Is Glut5. *J Biol Chem* 267: 14523-26
- Cataldo AM, Broadwell RD. 1986. Cytochemical identification of cerebral glycogen and glucose-6-phosphatase activity under normal and experimental conditions. II. Choroid plexus and ependymal epithelia, endothelia and pericytes. *J Neurocytol* 15: 511-24
- Champe PC, Harvey RA, Ferrier DR. 2008. *Biochemistry* pp. 69-82, 91-108, 109-116, 117-124, 125-136 and 137-144. London: Lippincott Williams & Wilkins.
- Chesler A, Himwich HE. 1944. Effect of insulin hypoglycemia on glycogen content of parts of the central nervous system of the dog. *Archives of Neurology and Psychiatry* 52: 114-16
- Choi HB, Gordon GR, Zhou N, Tai C, Rungta RL, et al. 2012. Metabolic communication between astrocytes and neurons via bicarbonate-responsive soluble adenylyl cyclase. *Neuron* 75: 1094-104
- Choi IY, Lee SP, Kim SG, Gruetter R. 2001. In vivo measurements of brain glucose transport using the reversible Michaelis-Menten model and simultaneous measurements of cerebral blood flow changes during hypoglycemia. *J Cerebr Blood F Met* 21: 653-63
- Choi IY, Seaquist ER, Gruetter R. 2003. Effect of hypoglycemia on brain glycogen metabolism in vivo. *J Neurosci Res* 72: 25-32
- Cruz NF, Dienel GA. 2002. High glycogen levels in brains of rats with minimal environmental stimuli: implications for metabolic contributions of working astrocytes. *J Cerebr Blood Flow Metab* 22: 1476-89
- Debernardi R, Pierre K, Lengacher S, Magistretti PJ, Pellerin L. 2003. Cell-specific expression pattern of monocarboxylate transporters in astrocytes and neurons observed in different mouse brain cortical cell cultures. *J Neurosci Res* 73: 141-55
- Deitmer JW, Schlue WR. 1989. An inwardly directed electrogenic sodium-bicarbonate co-transport in leech glial cells. *J Physiol* 411: 179-94
- Dienel GA. 2009. From molecules to networks : an introduction to cellular and molecular neuroscience, pp. 49-110. London: Academic Press
- Dienel GA, Cruz NF. 2004. Nutrition during brain activation: does cell-to-cell lactate shuttling contribute significantly to sweet and sour food for thought? *Neurochem Int* 45: 321-51
- Diggle CP, Shires M, Leitch D, Brooke D, Carr IM, et al. 2009. Ketohexokinase: Expression and Localization of the Principal Fructose-metabolizing Enzyme. *J Histochem Cytochem* 57: 763-74

- Domenech-Estevez E, Baloui H, Repond C, Rosafio K, Medard JJ, et al. 2015. Distribution of monocarboxylate transporters in the peripheral nervous system suggests putative roles in lactate shuttling and myelination. *J Neurosci* 35: 4151-6
- Franken P, Gip P, Hagiwara G, Ruby NF, Heller HC. 2003. Changes in brain glycogen after sleep deprivation vary with genotype. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* 285: R413-R19
- Gibbs ME, Anderson DG, Hertz L. 2006. Inhibition of glycogenolysis in astrocytes interrupts memory consolidation in young chickens. *Glia* 54: 214-22
- Gould GW, Holman GD. 1993. The glucose transporter family: structure, function and tissue-specific expression. *Biochem J* 295 (Pt 2): 329-41
- Halestrap AP, Price NT. 1999. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem J* 343 Pt 2: 281-99
- Halim ND, McFate T, Mohyeldin A, Okagaki P, Korotchkina LG, et al. 2010. Phosphorylation status of pyruvate dehydrogenase distinguishes metabolic phenotypes of cultured rat brain astrocytes and neurons. *Glia* 58: 1168-76
- Hassel B, Elsaïs A, Froland AS, Tauboll E, Gjerstad L, et al. 2015. Uptake and metabolism of fructose by rat neocortical cells in vivo and by isolated nerve terminals in vitro. *J Neurochem* 133: 572-81
- Hatazawa J, Ito M, Matsuzawa T, Ido T, Watanuki S. 1988. Measurement of the Ratio of Cerebral Oxygen-Consumption to Glucose-Utilization by Positron Emission Tomography - Its Consistency with the Values Determined by the Kety-Schmidt Method in Normal Volunteers. *J Cerebr Blood F Met* 8: 426-32
- Havel PJ. 2005. Dietary fructose: implications for dysregulation of energy homeostasis and lipid/carbohydrate metabolism. *Nutr Rev* 63: 133-57
- Heller BA, Ghidinelli M, Voelkl J, Einheber S, Smith R, et al. 2014. Functionally distinct PI 3-kinase pathways regulate myelination in the peripheral nervous system. *J Cell Biol* 204: 1219-36
- Herrero-Mendez A, Almeida A, Fernandez E, Maestre C, Moncada S, Bolanos JP. 2009. The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C-Cdh1. *Nature Cell Biology* 11: 747-U105
- Hoppe D, Chvatal A, Kettenmann H, Orkand RK, Ransom BR. 1991. Characteristics of Activity-Dependent Potassium Accumulation in Mammalian Peripheral-Nerve Invitro. *Brain Research* 552: 106-12
- Hwang JJ, Jiang L, Hamza M, Dai F, Belfort-DeAguiar R, et al. 2017. The human brain produces fructose from glucose. *JCI Insight* 2: e90508
- Izumi Y, Zorumski CF. 2009. Glial-neuronal interactions underlying fructose utilization in rat hippocampal slices. *Neuroscience* 161: 847-54
- Karagiannis A, Sylantyev S, Hadjihambi A, Hosford PS, Kasparov S, Gourine AV. 2016. Hemichannel-mediated release of lactate. *J Cerebr Blood F Met* 36: 1202-11
- Kidd GJ, Ohno N, Trapp BD. 2013. Biology of Schwann cells. *Handb Clin Neurol* 115: 55-79
- Kofuji P, Newman EA. 2004. Potassium buffering in the central nervous system. *Neuroscience* 129: 1045-56
- Kuffler SW, Potter DD. 1964. Glia in the Leech Central Nervous System: Physiological Properties and Neuron-Glia Relationship. *J Neurophysiol* 27: 290-320
- Lennertz RC, Medler KA, Bain JL, Wright DE, Stucky CL. 2011. Impaired sensory nerve function and axon morphology in mice with diabetic neuropathy. *J Neurophysiol* 106: 905-14

- Lerchundi R, Fernandez-Moncada I, Contreras-Baeza Y, Sotelo-Hitschfeld T, Machler P, et al. 2015. NH₄(+) triggers the release of astrocytic lactate via mitochondrial pyruvate shunting. *Proc Natl Acad Sci U S A* 112: 11090-5
- Lieberman EM, Abbott NJ, Hassan S. 1989. Evidence that glutamate mediates axon-to-Schwann cell signaling in the squid. *Glia* 2: 94-102
- Low FN. 1976. The perineurium and connective tissue of peripheral nerve. In *The Peripheral Nerve*, pp. 159-87. London: Chapman and Hall Ltd
- Maekawa K, Tanimoto T, Okada S, Suzuki T, Suzuki T, Yabe-Nishimura C. 2001. Expression of aldose reductase and sorbitol dehydrogenase genes in Schwann cells isolated from rat: effects of high glucose and osmotic stress. *Brain Res Mol Brain Res* 87: 251-6
- Magistretti PJ, Allaman I. 2015. A Cellular Perspective on Brain Energy Metabolism and Functional Imaging. *Neuron* 86: 883-901
- Magnani P, Cherian PV, Gould GW, Greene DA, Sima AA, Brosius FC, 3rd. 1996. Glucose transporters in rat peripheral nerve: paranodal expression of GLUT1 and GLUT3. *Metabolism* 45: 1466-73
- Mangia S, Giove F, Dinuzzo M. 2013. K⁺ homeostasis in the brain: a new role for glycogenolysis. *Neurochem Res* 38: 470-1
- Manty GJ, James DE, Devaskar SU. 1993. Jejunal Kidney Glucose Transporter Isoform (Glut-5) Is Expressed in the Human Blood-Brain-Barrier. *Endocrinology* 132: 35-40
- Meakin PJ, Fowler MJ, Rathbone AJ, Allen LM, Ransom BR, et al. 2007. Fructose metabolism in the adult mouse optic nerve, a central white matter tract. *J Cereb Blood Flow Metab* 27: 86-99
- Mizisin AP, Weerasuriya A. 2011. Homeostatic regulation of the endoneurial microenvironment during development, aging and in response to trauma, disease and toxic insult. *Acta Neuropathol* 121: 291-312
- NIDDK. 2013. Nerve damage (Diabetic neuropathies).
- Obrosova IG. 2009. Diabetes and the peripheral nerve. *Biochim Biophys Acta* 1792: 931-40
- Okamoto K, Wang WY, Rounds J, Chambers EA, Jacobs DO. 2001. ATP from glycolysis is required for normal sodium homeostasis in resting fast-twitch rodent skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism* 281: E479-E88
- Orte C, Lawrenson JG, Finn TM, Reid AR, Allt G. 1999. A comparison of blood-brain barrier and blood-nerve barrier endothelial cell markers. *Anat Embryol (Berl)* 199: 509-17
- Pellegrini G, Rossier C, Magistretti PJ, Martin JL. 1996. Cloning, localization and induction of mouse brain glycogen synthase. *Brain Res Mol Brain Res* 38: 191-9
- Pellerin L, Magistretti PJ. 1994. Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci U S A* 91: 10625-9
- Pellerin L, Pellegrini G, Bittar PG, Charnay Y, Bouras C, et al. 1998. Evidence supporting the existence of an activity-dependent astrocyte-neuron lactate shuttle. *Dev Neurosci* 20: 291-9
- Pfeiffer-Guglielmi B, Fleckenstein B, Jung G, Hamprecht B. 2003. Immunocytochemical localization of glycogen phosphorylase isozymes in rat nervous tissues by using isozyme-specific antibodies. *J Neurochem* 85: 73-81
- Pfeiffer-Guglielmi B, Francke M, Reichenbach A, Hamprecht B. 2007. Glycogen phosphorylase isozymes and energy metabolism in the rat peripheral nervous system--an immunocytochemical study. *Brain Res* 1136: 20-7

- Pinnacle technology i. 2017. Lactate biosensor.
- Poulsom R, Mirrlees DJ, Earl DCN, Heath H. 1983. The Effects of an Aldose Reductase Inhibitor Upon the Sorbitol Pathway, Fructose-1-Phosphate and Lactate in the Retina and Nerve of Streptozotocin-Diabetic Rats. *Experimental Eye Research* 36: 751-60
- Rafiki A, Boulland JL, Halestrap AP, Ottersen OP, Bergersen L. 2003. Highly differential expression of the monocarboxylate transporters MCT2 and MCT4 in the developing rat brain. *Neuroscience* 122: 677-88
- Ramos M, del Arco A, Pardo B, Martinez-Serrano A, Martinez-Morales JR, et al. 2003. Developmental changes in the Ca²⁺-regulated mitochondrial aspartate-glutamate carrier aralar1 in brain and prominent expression in the spinal cord. *Brain Res Dev Brain Res* 143: 33-46
- Rosengarten H. 1970. Glycogen level and oxygen consumption of rabbit tissue slices under ethyl ether and halothane anesthesia. *Agressologie* 11: 159-62
- Rossen R, Kabat H, Anderson JP. 1943. Acute arrest of cerebral circulation in man. *Archives of Neurology and Psychiatry* 50: 510-28
- Sabri MI, Ochs S. 1971. Inhibition of glyceraldehyde-3-phosphate dehydrogenase in mammalian nerve by iodoacetic acid. *J Neurochem* 18: 1509-14
- Saitoh M, Okada Y, Nabetani M. 1994. Effect of Mannose, Fructose and Lactate on the Preservation of Synaptic Potentials in Hippocampal Slices. *Neuroscience Letters* 171: 125-28
- Schmalbruch H. 1986. Fiber composition of the rat sciatic nerve. *Anat Rec* 215: 71-81
- Sepp M, Sokolova N, Jugai S, Mandel M, Peterson P, Vendelin M. 2014. Tight Coupling of Na⁺/K⁺-ATPase with Glycolysis Demonstrated in Permeabilized Rat Cardiomyocytes. *Plos One* 9
- Shulman RG, Bloch G, Rothman DL. 1995. In-Vivo Regulation of Muscle Glycogen-Synthase and the Control of Glycogen-Synthesis. *Proceedings of the National Academy of Sciences of the United States of America* 92: 8535-42
- Squire L, Bloom FE, Lac S, Ghosh A, Spitzer NC. 2013. *Fundamental Neuroscience*. pp. 3-40. Oxford: Academic Press.
- Stecker MM, Stevenson MR. 2015. Anoxia-induced changes in optimal substrate for peripheral nerve. *Neuroscience* 284: 653-67
- Sun SZ, Empie MW. 2012. Fructose metabolism in humans - what isotopic tracer studies tell us. *Nutr Metab (Lond)* 9: 89
- Swanson RA, Choi DW. 1993. Glial glycogen stores affect neuronal survival during glucose deprivation in vitro. *J Cereb Blood Flow Metab* 13: 162-9
- Takebe K, Nio-Kobayashi J, Takahashi-Iwanaga H, Iwanaga T. 2008. Histochemical demonstration of a monocarboxylate transporter in the mouse perineurium with special reference to GLUT1. *Biomed Res* 29: 297-306
- Tappy L, Le KA. 2010. Metabolic effects of fructose and the worldwide increase in obesity. *Physiol Rev* 90: 23-46
- Tashiro S. 1922. Studies on alkaligenesis in tissues I. Ammonia production in the nerve fiber during excitations. *Am J Physiol* 60: 519-43
- Tekkok SB, Brown AM, Ransom BR. 2003. Axon function persists during anoxia in mammalian white matter. *J Cereb Blood Flow Metab* 23: 1340-7
- Tsacopoulos M, Veuthey AL, Saravelos SG, Perrottet P, Tsoupras G. 1994. Glial cells transform glucose to alanine, which fuels the neurons in the honeybee retina. *J Neurosci* 14: 1339-51
- Ueno M, Nishi N, Nakagawa T, Chiba Y, Tsukamoto I, et al. 2014. Immunoreactivity of glucose transporter 5 is located in epithelial cells of the choroid plexus and ependymal cells. *Neuroscience* 260: 149-57

- Vannucci SJ, Maher F, Simpson IA. 1997. Glucose transporter proteins in brain: delivery of glucose to neurons and glia. *Glia* 21: 2-21
- Vannucci SJ, Simpson IA. 2003. Developmental switch in brain nutrient transporter expression in the rat. *Am J Physiol Endocrinol Metab* 285: E1127-34
- Vega C, Martiel JL, Drouhault D, Burckhart MF, Coles JA. 2003. Uptake of locally applied deoxyglucose, glucose and lactate by axons and Schwann cells of rat vagus nerve. *J Physiol* 546: 551-64
- Vega C, Poitry-Yamate CL, Jirounek P, Tsacopoulos M, Coles JA. 1998. Lactate is released and taken up by isolated rabbit vagus nerve during aerobic metabolism. *J Neurochem* 71: 330-7
- Viader A, Golden JP, Baloh RH, Schmidt RE, Hunter DA, Milbrandt J. 2011. Schwann cell mitochondrial metabolism supports long-term axonal survival and peripheral nerve function. *J Neurosci* 31: 10128-40
- Wada H, Okada Y, Uzuo T, Nakamura H. 1998. The effects of glucose, mannose, fructose and lactate on the preservation of neural activity in the hippocampal slices from the guinea pig. *Brain Res* 788: 144-50
- Wahjudi PN, Patterson ME, Lim S, Yee JK, Mao CS, Lee WNP. 2010. Measurement of glucose and fructose in clinical samples using gas chromatography/mass spectrometry. *Clinical Biochemistry* 43: 198-207
- Walz W, Mukerji S. 1988. Lactate release from cultured astrocytes and neurons: a comparison. *Glia* 1: 366-70
- Weerasuriya A, Mizisin AP. 2011. The Blood-Nerve Barrier: Structure and Functional Significance. *Methods Mol Biol* 686: 149-73
- Wender R, Brown AM, Fern R, Swanson RA, Farrell K, Ransom BR. 2000. Astrocytic glycogen influences axon function and survival during glucose deprivation in central white matter. *J Neurosci* 20: 6804-10
- Whatley SA, Hall C, Lim L. 1981. Hypothalamic neurons in dissociated cell culture: the mechanism of increased survival times in the presence of non-neuronal cells. *J Neurochem* 36: 2052-6
- Williams LR, Leggett RW. 1989. Reference Values for Resting Blood-Flow to Organs of Man. *Clinical Physics and Physiological Measurement* 10: 187-217
- Yamane K, Yokono K, Okada Y. 2000. Anaerobic glycolysis is crucial for the maintenance of neural activity in guinea pig hippocampal slices. *Journal of Neuroscience Methods* 103: 163-71