

A Study of Mitochondrial Dynamics and Glycosylation Events in PC12 Cells and Neurons



Trinity College Dublin

Coláiste na Tríonóide, Baile Átha Cliath

The University of Dublin

Thesis submitted to the University of Dublin for the degree of Doctor of
Philosophy

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Submitted August 2017

Declaration

I declare that this thesis has not been submitted as an exercise for any degree at this or any other university and is entirely my own work, with the following exceptions:

- Measurement of ATP levels in primary cortical neurons in Fig 3.24 (B), which was carried out by Stephen Quinn.
- Generation of stable PC12 cells over expressing wild type and mutant (A52T) α -synculein, which was carried out in conjunction with Natalie Adlesic.

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Darren O Hara

Acknowledgements

After an incredible four year experience has come to an end and it's heartening to think I could fill most of the pages of this book with people who deserve thanks. First and foremost, enormous credit is due to my supervisor Dr. Gavin Davey for his support, guidance and for putting up with my all too regular knocks on your door over the past few years. You've always been quick to answer the simplest of questions and guide me in the right direction while giving me the freedom to come to my own conclusions. I will be forever grateful for this opportunity, Thank you very much Gav.

I would like to thank the Irish Research Council for funding this project and for their funding of an enormous variety of essential research projects in this country.

To all the members of the Davey lab, past and present, who have made this the most enjoyable working environment I've ever had the privilege to be a part of. Jer "The Silver Fox" Hayes; whether it's been a quick after-work pint(s), a discussion on the weekends golf, advice on how to make money on the NFL or sometimes even science, you've always been there for me. I'm lucky enough to call you a mentor and a friend, thank you. Natalie, for an ABBA/Westlife sing-a-long, for a discussion on the moral complexities of life, for a couch to sleep on, for David (<3) and for O-GlcNAc, thank you. Laura, I don't even know where to start, so I'm not gonna say anything, thank you. Ryan, for the long hours spent in the dungeon together and the scientific discussion/rants over several pints, thank you. Aisling, Nicoleta, Cecile, Gerard, Olga, Spanish Laura, Cavan Laura, Andy, Yong-Jing, Sahar, Andrew; you've all provided me with laughs, advice and sweets over the years, thank you all.

To all the other members of the 5th floor reading room for making this a place where it's far easier to have a coffee/glass of wine and a chat than to actually get some work done. Niki, Paul, Jenni and Nidhi; we may not have solved any scientific problems but we solved plenty of world problems hidden away round

the corner. Nial, Simon and Ray; I'll be forever thankful for having a few blokes to talk football with after lunch (there was a nail bar set up after ye left!!). Roisin, Katie, Jess, Nadine, Peter and everyone else; cheers for all the lunchtime bantz and many late nights; can't wait for the Barbados reunion.

To all my housemates over the years who've made me look forward to getting home after the long days. Reynolds, Conall, Aonghus, Hegz and Big Mike; thanks for taking my mind off science, providing me with biscuits (bisciins) and enjoying an ol' episode of University Challenge/The Chase. Thanks to all my other friends in Dublin, Sligo and further afield for their support and acting interested when I tell them what I'm up to.

To Jane, you've had to put up with as much as anyone and smiled (or ran) through it all. Your support, encouragement, work ethic and attitude to life has motivated me every step of the way and helped me through the toughest times. You can now call yourself an "Officer of the Law and Mitochondrial Dynamics". Thank you and Love you.

Finally, and most importantly, to Mum and Dad. For as long as I can remember, you've been rocks of support for me; encouraging me in everything I've done, supporting my ambitions and being there whenever I needed anything. Never has that been truer than throughout the past 4 years. I genuinely couldn't have done this without you. I can only hope I've made you proud.

Summary

Mitochondrial dysfunction is recognised as a hallmark of many neurodegenerative diseases. Altered electron transport chain (ETC) complex activities, mitochondrial fusion/fission dynamics, mitochondrial motility kinetics and quality control of mitochondrial biogenesis have been implicated in the pathogenesis of Parkinson's Disease (PD) and Alzheimer's Disease (AD). However, further elucidation of the mechanisms that underlie dysfunctions in mitochondrial dynamics is required. This study characterizes the effect of electron transport chain (ETC) inhibition on mitochondrial dynamics in differentiated PC12 cells and primary cortical neurons. Inhibition of any of the ETC complexes, ATP synthase or dissipation of the mitochondrial membrane potential was sufficient to completely abolish mitochondrial fusion in differentiated PC12 cells. This effect is dependent on a post-translational modification that cleaves OPA1, the protein responsible for inner mitochondrial membrane fusion, to a shorter form, thus inhibiting fusion. It was also found that complex I inhibition has a threshold effect on mitochondrial fusion in primary cortical neurons as 55% inhibition of complex I completely inhibits mitochondrial fusion while 53% inhibition has no significant effect. This effect also correlated with a mitochondrial membrane potential dependent cleavage of Opa1. Mutations in, and abnormal accumulation of, the presynaptic protein α -synuclein are highly correlated with the pathogenesis of PD. Accumulations of this protein have been shown to inhibit complex I. Overexpression of both wild-type and mutant forms of α -synculein were found to decrease mitochondrial fusion in differentiated PC12 cells, suggesting that α -synculein dependent changes in fusion/fission dynamics may be a complicating factor in PD.

Altered substrate utilization and O-GlcNAcylation have also been linked to the progression of some neurodegenerative diseases. In this study, inhibition of fatty acid oxidation and pyruvate transport into the mitochondrion decreased mitochondrial fusion in differentiated PC12 cells. O-GlcNAcylation is a post-translational modification that attaches O-GlcNAc moieties to cytoplasmic,

nuclear and mitochondrial proteins. Investigation into acute increases in O-GlcNAcylation, induced by inhibition or knockdown of O-GlcNAcase, revealed decreases in mitochondrial fusion, elongated mitochondrial morphology, increased respiration rates and increased complex I activity. A decrease in the level of O-GlcNAcylation, induced by inhibition or knockdown of O-GlcNAc transferase, resulted in increased mitochondrial fusion, decreased mitochondrial membrane potential, fragmented mitochondrial morphology and decreased respiration rates in differentiated PC12 cells. Knockdown of O-GlcNAc transferase also resulted in increased ROS production. The results presented in this thesis point to altered complex I activity having an important role in mitochondrial dysfunction and subsequently, mitochondrial dynamics in the brain. Identification of compounds which can regulate complex I activity, may be able to restore balance to mitochondrial dynamics and halt or slow the progression of neurodegenerative diseases.

Finally, this study demonstrated it is possible to visualize the cell surface glycome of primary cortical neurons by metabolic labelling. Three modified azido sugars were incorporated into the cell surface glycome and labelled using click chemistry. Super resolution microscopy identified intriguing heavily sialylated structures on the cell surface of neuronal projections. In addition to this, use of inhibitors of various glycosyltransferases allowed for some elucidation of the make-up of the neuronal cell surface glycome which was found to be predominantly *N*-glycans. These findings may help to identify glycans important for neuronal differentiation and synaptic transmission.

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Abbreviations

3-NP	3-Nitropropionic acid
2,4-DNP	2,4-Dinitrophenol
$\Delta\psi_m$	Mitochondrial Membrane Potential
A53T	Alanine 53 Threonine
A β	Beta-amyloid
ACAD	Acyl Co-A dehydrogenase
AChE	Acetylcholinesterase
ACOX	Acetyl Co-A oxidase
AD	Alzheimer's Disease
ADP	Adenosine diphosphate
ALS	Amyotrophic Lateral Sclerosis
ANT	Adenine nucleotide translocase
ANOVA	Analysis of Variance
APP	Amyloid precursor protein
Ara-C	Cytosine β -D-arabinofuranoside
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
CACT	Carnitine acyl-carnitine translocase
CMT	Charcot-Marie-Tooth neuropathy
CPT1	Carnitine palmitoyltransferase 1
CREB	cAMP response element binding protein
CSF	Cerebro-spinal fluid
DA	Dopamine
DCA	Dichloroacetate
DNA	Deoxyribonucleic Acid
Drp1	Dynamin-related protein 1
ECAR	Extracellular acidification rate
ECL	Enhanced chemi-luminescence
ER	Endoplasmic Reticulum
ETC	Electron Transport Chain

ETF	Electron Transferring Protein
FADH ₂	Flavin Adenine Dinucleotide (reduced)
FBS	Fetal Bovine Serum
FCCP	Carbonyl Cyanide-4-Phenylhydrazone
FMN	Flavin Mononucleotide
GAG	Glycosaminoglycan
GalNAc	N-Acetylgalactosamine
GBP	Glycan Binding protein
GlcNAc	N-Acetylglucosamine
H ₂ DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H ₂ O ₂	Hydrogen Peroxide
HAT	Histone Acetyltransferase
HBP	Hexosamine Biosynthetic Pathway
HD	Huntington's Disease
HR2	Heptad Repeat Region
INF2	Formin 2
IP3 kinase	Phosphatidylinositol 3-kinase
iPSc	Induced Pluripotent Stem Cell
KCN	Potassium Cyanide
KO	Knockout
LD	Lipid Droplet
LHON	Leber's Hereditary Optic Neuropathy
LLO	Lipid Linked Oligosaccharide
LS	Leigh Syndrome
LTP	Long Term Potentiation
MAM	Mitochondrial Associated Membrane
ManNAc	N-Acetylmannosamine
Mao-B	Monoamine Oxidase B
MAPK	Mitogen-activated Protein Kinase
MCI	Mild Cognitive Impairment
MELAS	Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like Episodes

Mff	Mitochondrial Fission Factor
Mfn	Mitofusin
MIM	Mitochondrial Inner Membrane
MIP	Mitochondrial Intermediate Peptidase
MOM	Mitochondrial Outer Membrane
MPC	Mitochondrial Pyruvate Carrier
MPP ⁺	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	Mitochondrial Deoxyribonucleic Acid
nAChR	Nicotinic Acetylcholine Receptor
NADH	Nicotinamide Adenine Dinucleotide (reduced)
NCAM	Neural Cell Adhesion Molecule
NFT	Neurofibrillary Tangles
NGF	Nerve Growth Factor
NO	Nitric Oxide
NRF1	Nuclear Respiratory Factor 1
NSM	Non-Synaptic Mitochondria
O ₂ ^{•-}	Superoxide Anion
-OH	Hydroxyl Radical
OCR	Oxygen Consumption Rate
O-GlcNAc	O-linked N-acetylglucosamine
OGA	O-GlcNAcase
OGT	O-GlcNAc Transferase
Opa1	Optic Atrophy 1
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate Buffered Saline
PD	Parkinson's Disease
PDC	Pyruvate Dehydrogenase Complex
PDH	Pyruvate Dehydrogenase
PDK	Pyruvate Dehydrogenase Kinase
PDP	Pyruvate Dehydrogenase Phosphatase
PKA	Protein Kinase A

PMF	Proton Motive Force
PTP	Permeability Transition Pore
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RCR	Respiratory Control Ratio
ROI	Region of Interest
ROS	Reactive Oxygen Species
SCA	Spinocerebellar Ataxia
SEM	Standard Error of the Mean
SN	Substantia Nigra
SNARE	Soluble NSF Attachment Protein Receptor
SOD	Superoxide Dismutase
TCA	Tricarboxylic Acid
TOM	Translocase of the Outer Membrane
TPR	Tetratricopeptide repeat
TT04	St045849
UCP	Uncoupling Protein
WWS	Walker Warburg Syndrome

Chapter 1

General Introduction

1.1 Introduction

Mitochondria are vital organelles, which perform a wide range of functions that have been designated the “powerhouses of the cell” and have been implicated in many different diseases as research has intensified over the past number of years. A primary function of mitochondria is to provide each cell with energy in the form of adenosine triphosphate (ATP) which is produced as a result of two catabolic processes (Canbäck and Kurland, 2003). Firstly, glucose is converted to pyruvate in the cytoplasm through the process of glycolysis which encompasses a series of enzymatic reactions with each intermediate providing potential entry points to the glycolytic process (Racker, 1974). Pyruvate is be converted to lactic acid unless oxygen is present, whereby pyruvate is transported to the mitochondria via mitochondrial pyruvate carriers and oxidized to CO₂ and H₂O by a process known as oxidative phosphorylation (Racker, 1974, Saraste, 1999).

This dynamic organelle has sparked the interest of scientists for many years who have sought to understand its mechanistic functions, not only because of its importance in controlling cellular homeostasis but also because of its role in a wide range of diseases. Research on the mitochondrion has resulted a large number of Nobel prizes from Warburg and Krebs who carried out some ground-breaking research on cellular respiration in the first half of the 20th century to more recent advancements surrounding the process of oxidative phosphorylation and the enzymes and protein complexes that allow it to happen (Fearnley et al., 2006, Mitchell, 1974). Mitochondrial diseases rank among the most common inherited disorders and can arise as a result of mutations in mitochondrial DNA (mtDNA) or nuclear DNA generally causing diseases such as Leber’s hereditary optic neuropathy (LHON), Mitochondrial encephalopathy, lactic acidosis, stroke-like episodes (MELAS) or Leigh’s Syndrome (LS) (Ylikallio and Suomalainen, 2012). A majority of the mutations associated with mitochondrial disorders are in genes coding for subunits of the oxidative phosphorylation (OXPHOS) machinery or assembly factors for these subunits although diseases can also arise through

impaired mtDNA maintenance and many more indirect mechanisms. Because of the distribution of mitochondria in cells of every tissue (except red blood cells), it is a challenge to develop therapies that effectively treat mitochondria-related disorders. Current therapies focus on alleviating the severity of symptoms (Nunnari and Suomalainen, 2012) (Suomalainen, 2011). As well as the obvious metabolic disorders resulting from damage to the respiratory chain, mitochondrial dysfunction has recently been shown to play an important role in many neurodegenerative diseases (Lin and Beal, 2006) and cancer (Wallace, 2012).

The notion that mitochondria are of bacterial origin was first put forward by Portier in 1918 but it was almost half a century later before the scientific community seriously discussed this possibility. It wasn't until 1963 when it was shown that each mitochondrion has its own genome (Nass and Nass, 1963) that the debate was reignited and further efforts were made to establish the evolutionary origins of the mitochondrion. Today, we know that the likely origin of the mitochondrion is an organism similar to the intracellular parasite *Rickettsia prowazekii* that causes typhus. Sequencing of the entire genome of the parasite shows no genes required for anaerobic glycolysis but a complete set of genes coding for components of the tricarboxylic acid (TCA) cycle and components of the electron transport chain (Andersson et al., 1998). Despite much evidence linking mitochondria to a bacterial origin, it should be noted that human mitochondria contain a circular genome of about 17 kilobases (kb) in size, which compares with a bacterial genome ranging from 160kb to 13 megabases (Koonin, 2009) but biochemical evidence suggests that the bacterial genome would have had a similar composition to today at the time of a symbiotic event (Nisbet and Sleep, 2001). The mystery of the reduction in size of the mitochondrial genome since an endosymbiotic event can be explained by a portion of the genome being lost during the event itself while a greater portion of coding regions are likely to have translocated to the nucleus in a process known as endosymbiotic gene transfer (Timmis et al., 2004). Thus, the highly efficient mitochondria evident today, which produce large amounts of ATP, synthesize the vast array of subunits and

enzymes required for the process from a combination of mtDNA and nuclear DNA despite a common prokaryotic background.

Mitochondria are most renowned for ATP production through the process of OXPHOS where electron carriers donate electrons to members of the electron transport chain (ETC) generating a proton motive force across the mitochondrial inner membrane (MIM) which is used to generate ATP by the phosphorylation of adenosine diphosphate (ADP) (Saraste, 1999). However, mitochondria are also involved in a variety of other important cellular functions including the use of mitochondrial membrane potential generated during OXPHOS to import other essential mitochondrial proteins (Neupert and Herrmann, 2007) as well as producing reactive oxygen species (ROS) which can have damaging effects on many organelles and biomolecules within the cell (Murphy, 2009). Alternatively, ROS can also benefit the cell by responding to the activation of stress signalling pathways (ERK/MAPK) by controlling cell proliferation and differentiation (Weinberg and Hamanaka, 2010). Mitochondria also contain many essential proteins for the lipid biosynthetic pathway, including the fatty-acid β -oxidation pathway which is an important consideration for the current study, as well as providing components for the synthesis of metals such as heme and Fe-S clusters which are essential for hemoglobin biosynthesis within the body (Lill and Mühlenhoff, 2008). Mitochondria are also key regulators of cell death playing a central role in both apoptotic and necrotic cell death. Apoptotic cell death is initiated through the permeabilization of the mitochondrial outer membrane (MOM) releasing several proteins, such as cytochrome c, AIF and EndoG, which perform vital functions within the mitochondrion but are toxic in the cytosol (Whelan et al., 2010a). The MOM permeabilization is promoted by pro cell death members of the BCL-2 family of proteins including BAX and BAK which can be inhibited by other family members including Bcl-2 and Bcl-xL which will be discussed further at a later point (Chipuk et al., 2010). Finally, the mitochondrion has an essential role in Ca^{2+} homeostasis by buffering Ca^{2+} flux from the endoplasmic reticulum (ER) and plasma membrane (Rizzuto et al., 2012). This

function, is particularly relevant to this study as Ca^{2+} is essential for neurotransmitter synthesis, release and the regulation of synaptic plasticity.

1.2 Mitochondrial Bioenergetics

Mitochondria produce up to 90% of cellular ATP used to carry out a myriad of functions within the cell, the majority of which is generated through OXPHOS (Saraste, 1999) (Benard, 2008). This process begins after the major phase of cellular catabolism, which produces pyruvate and fatty acids through the processes of glycolysis and lipolysis. These smaller molecules are transported across the mitochondrial membrane by specific transport proteins where they are further degraded into reduced forms of nicotinamide adenine dinucleotide (NADH) and/or flavin adenine dinucleotide (FADH₂) by β -oxidation and the Krebs cycle. These reduced equivalents are then oxidized and the liberated electrons are passed along a series of mobile carriers known as the ETC. The ETC is located in the inner mitochondrial membrane and consists of 4 protein complexes (complexes I-IV) and 2 electron carriers (coenzyme Q and cytochrome C). Each complex is composed of a number of subunits encoded by a combination of nuclear DNA and mtDNA, with the exception of complex II, which is encoded by nuclear DNA only. When an energy substrate is present, the transfer of electrons from complex I (or CII) to complex IV results in the extrusion of protons against their concentration gradient from the mitochondrial matrix to the inter-membrane space which generates an electrochemical gradient or protonmotive force (PMF). This gradient is then used by ATP synthase (or complex V) to phosphorylate ADP, thus producing ATP, while the electrons are used to reduce molecular oxygen to water. The newly formed ATP is translocated to the cytosol by adenine nucleotide translocase (ANT) where it used to meet the energy requirements of the cell. This method of coupling respiration to the synthesis of ATP is known as OXPHOS (Benard et al., 2011).

Complex I (CI), or NADH ubiquinone oxidoreducataase, is the largest of the complexes within the ETC and consists of 45 subunit proteins, 7 of which are encoded by the mitochondrial genome (Sazanov, 2015). CI is an L-shaped complex with one arm in the plane of the inner mitochondrial membrane and the other lying adjacent to it, protruding into the mitochondrial matrix (Carroll et al.,

2003). It catalyzes the transfer of electrons from NADH to a non-covalently bound flavin mononucleotide (FMN), then through a series of eight iron-sulfur (Fe-S) clusters to the terminal acceptor, ubiquinone (or Coenzyme Q) (Grigorieff, 1999). This transfer of 2 electrons from NADH to the mobile coenzyme Q results in the translocation of 4 protons across the inner membrane (Wikström, 1984). Coenzyme Q also accepts electrons from Complex II (CII), or succinate-ubiquinone oxidoreductase, which is the smallest of the mammalian respiratory chain complexes and has a dual function as a component of the ETC and as an enzyme for the TCA cycle owing to its location where it straddles the inner mitochondrial membrane. It consists of a peripheral domain, which projects into the matrix and is composed of a flavoprotein and an iron-sulfur subunit. The flavoprotein is flanked by two membrane bound anchor proteins and contains the active site of the enzyme containing FAD. Thus, electrons derived from the oxidation of succinate can be directly transferred to the ubiquinone pool and on to complex III (Ackrell, 2002).

Complex III (CIII), or Cytochrome bc_1 , is the middle component of the respiratory chain whose primary function is to transfer electrons from coenzyme Q to cytochrome c and to further generate a proton gradient across the mitochondrial inner membrane. CIII exists as dimer with each monomer composed of 11 protein subunits with a total mass of around 240 kDa (Iwata et al., 1998). Ten of these subunits are encoded by nuclear DNA and one, cytochrome b , is encoded by the mitochondrial genome (Donati et al., 2007). Cytochrome c is a small, mobile electron carrier whose reduction by $CoQH_2$ is mediated by CIII. During this “Q cycle”, $CoQH_2$ undergoes two cycles of reoxidation, which results in the reduction of two cytochrome c proteins and the translocation of 4 protons across the inner mitochondrial membrane (Barel et al., 2008). The Q-cycle begins when ubiquinol is oxidized to ubiquinone at the Q_0 site and 2 electrons are fed into a high-potential and low-potential chain (Osyczka et al., 2005). One electron is transferred to the Fe-S cluster of the Rieske protein and on to soluble cytochrome c via the heme of cytochrome c_1 resulting in the release of two protons into the intramembrane space. The other electron enters the low potential chain and is transferred onto

ubiquinone bound to the Q_i site forming a stabilized semiquinone. The cycle is completed by the oxidation of a second ubiquinol at the Q_o site resulting in the reduction of a second cytochrome *c* and reduction of the semiquinone to ubiquinol at the Q_i site (Bleier and Dröse, 2013).

Complex IV (CIV), or Cytochrome C Oxidase, is the terminal enzyme in the ETC where molecular oxygen is reduced to water. CIV consists of 13 subunits that form a monomer of 200 kDa but it often exists as a dimeric unit. Three of these subunits are encoded by the mitochondrial genome and these represent almost 60% of the total mass of the complex and play an extremely important role in proton pumping and the transfer of electrons to oxygen (Carr and Winge, 2003). The complete dimer contains four redox centres; cytochromes a and a_3 , a copper atom called Cu_B and a copper atom pair called the Cu_a centre. Cytochrome a_3 and Cu_B form a complex and it is here that four electrons from 4 cytochrome *c* proteins are used to reduce one oxygen molecule to two water molecules. As well as the 4 protons used to make water molecules, 4 protons are translocated across the inner mitochondrial membrane, strengthening the protonmotive force (Beinert, 1995). In total, for every electron pair transported down the ETC, 10 protons are translocated across the inner mitochondrial membrane against their gradient.

It was initially thought that these four complexes were randomly distributed throughout the inner mitochondrial membrane and that CoQ and Cytochrome *c* acted by shuttling electrons to the various complexes within the chain. This is known as the fluid model, but in recent years an alternative hypothesis has been suggested whereby the complexes are formed into larger structures, known as supercomplexes, within the membrane to allow for the quick and efficient trafficking of electrons (Lenaz et al., 2007). The evidence for the supercomplex model comes from blue native gel electrophoresis showing the co-migration of different ETC complexes or using density gradients and centrifugation (Acín-Pérez et al., 2008). Supercomplexes most commonly isolated after solubilisation using mild detergents are complex III with complex I and/or complex IV. Indeed, a 3D reconstruction of a $I_1III_2IV_1$ supercomplex shows a proximity of CoQ and

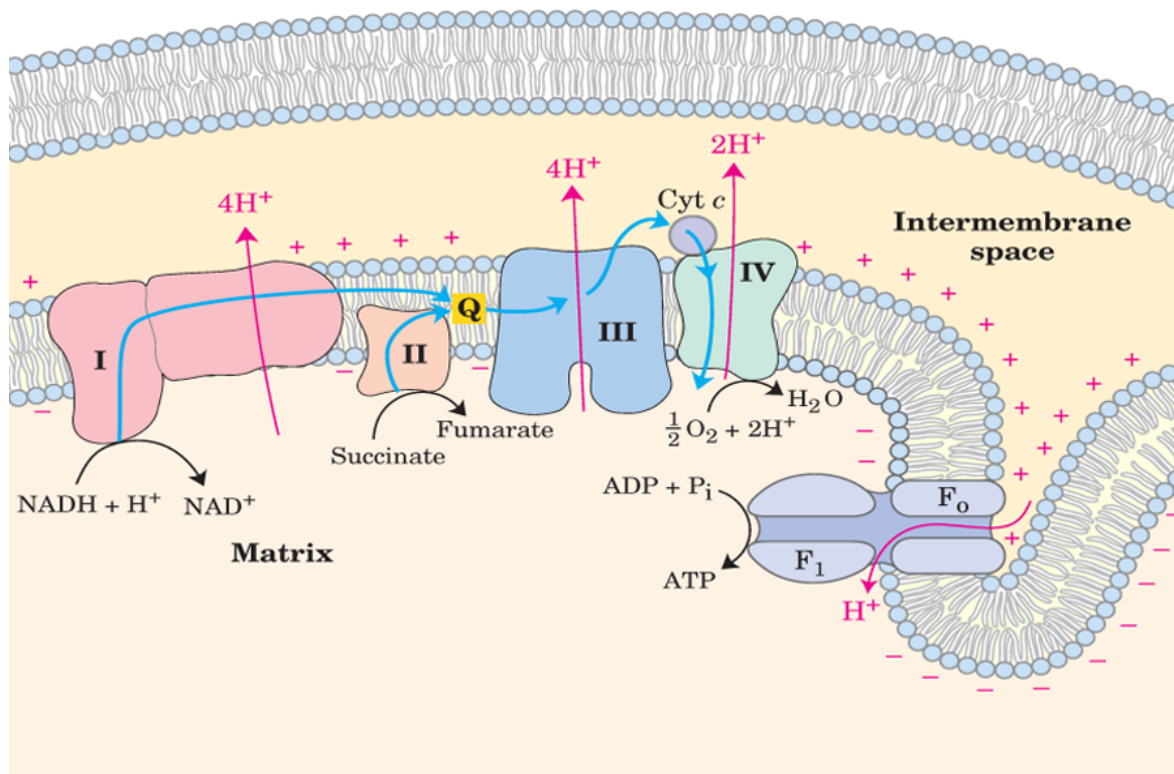


Figure 1.1 – Components of the Electron Transport Chain. Electrons are donated from NADH to Complex I which uses the free energy from the reduction of Coenzyme Q (Q) to transfer 4 protons (H⁺) out of the matrix, increasing the electrochemical gradient. Complex II oxidizes succinate, whilst reducing FAD, which reduces Q. Coenzyme Q then passes electrons to Complex III, which reduces cytochrome C (Cyt c) resulting in the transfer of 4 more protons across the inner mitochondrial membrane, again increasing the electrochemical gradient. Complex IV accepts electrons from Cyt c and reduces molecular oxygen to water whilst translocating a further 2 protons across the membrane. Finally, ATP synthase (F₀/F₁ ATPase) utilizes the proton motive force created by the ETC to produce ATP from ADP and inorganic phosphate in a process known as oxidative phosphorylation. Taken from Champe et al. (2005).

Cytochrome *c* binding sites suggesting efficient electron transfer and this structure has been obtained using single-particle cryo-electron microscopy (Wu et al., 2016). The biological benefits of supercomplex formation include a reduction in ROS generation, an increase in the catalytic activity of individual components and an increase in the efficiency of electron transfer through substrate channelling (Maranzana et al., 2013, Moreno-Lastres et al., 2012). Interestingly it has recently been shown that an intermediate between the fluid and supercomplex models exists whereby ubiquinone/ubiquinol and cytochrome *c* exist in pools within the mitochondria which are freely exchanged between complexes within supercomplexes (Blaza et al., 2014). It has also been shown that ATP synthase exists in dimer rows along the curved mitochondrial inner membrane showing that supramolecular organization of mitochondrial proteins is beneficial for the process of oxidative phosphorylation (Vonck and Schäfer, 2009).

1.2.1 Oxidative Phosphorylation (OXPHOS)

ATP Synthase, or complex V, is a membrane bound complex that couples the transmembrane proton-motive force to the synthesis of ATP. It is composed of 2 main structural domains; an F_1 domain, which is globular in shape and extends into the mitochondrial matrix, consists of an assembly of subunits α , β , γ , δ and ϵ in the ratio 3:3:1:1:1. The foot of this F_1 subunit makes extensive contact with the F_0 subunit, which is buried in the membrane and contains a ring of c-subunits (Fearnley et al., 2006). These two domains are linked together by a central stalk and peripheral stalk; the central stalk extends out from the centre of the c-subunit ring of F_0 . As the passage of protons through the channels drives the clockwise rotation of the ring, it also drives rotation of the central stalk, which allows each of the 3 catalytic sites on the F_1 subunit to go through a series of conformational changes. These various conformational states allow the binding of ADP and phosphate and subsequent formation and release of ATP (Walker and Dickson, 2006). Although it has not yet been fully established, it is believed that the movement of 4 protons is enough to rotate the stalk enough to produce a single ATP molecule (Yoshida et al., 2001). A more recent discovery has identified a role for ATP synthase in the formation of the permeability transition pore (PTP)

which is associated with the activation of the cell death pathway. Cyclophilin D (a central regulator of the PTP) binds the lateral stalk resulting in partial inhibition of ATP synthase activity followed by dimers of the complex being incorporated into lipid bilayers and forming Ca^{2+} -activated channels with the key features of the PTP (Giorgio et al., 2013).

Thus, it has been established that the critical step for production of ATP is the generation of the proton-motive force (PMF), across the inner mitochondrial membrane, which reaches a maximal value of between 180 and 220 mV and confers a net negative charge to the mitochondrial matrix (Brand and Nicholls, 2011). The PMF is comprised to two separate elements; an electrical potential ($\Delta\Psi$) and a chemical potential ($\Delta\mu_{\text{H}^+}$). At physiological states, mitochondria alternate between two energy production states; state 3 and state 4. During state 3, the rate of respiration (ie. flow of electrons through the respiratory chain) is fast and ATP is produced leading to a lower mitochondrial membrane potential ($\Delta\Psi_{\text{m}}$). During state 4, the rate of respiration is slow and ATP is not produced leading to a higher PMF. As a consequence, it is thought that mitochondria only produce ATP at an intermediate state somewhere between state 3 and 4 in a working mitochondrial system *in vivo* (Benard et al., 2011).

The respiratory control ratio (RCR) is the respiration in state 3 divided by that in state 4 and allows us to quantify the degree of coupling between electron transport and ATP synthesis. A high RCR value shows that mitochondria have a high capacity for ATP production and a low proton leak but this is not necessarily indicative of healthy mitochondria as certain tissues, such as brown adipose tissue, require a high level of proton leak for heat generation (Brand and Nicholls, 2011). A low RCR shows that there is a low degree of coupling and that there is a large amount of proton leak back into the matrix through a route other than ATP synthase. Proton leak is necessary for the function in most tissues and is facilitated by a group of uncoupling proteins (UCPs), such as UCP1, which is most highly expressed in brown adipose tissue as mentioned above. By uncoupling the mitochondria, the energy generated by the flow of electrons is no longer

harnessed to drive ATP production and is instead lost as heat, a property utilized by many mammals in hibernation (Nicholls and Rial, 1999) (Adams et al., 2008) (Cannon and Nedergaard, 2011). Thus, the RCR ratio is strongly influenced by almost every functional aspect of oxidative phosphorylation, most notably the proton-motive force, suggesting that any slight alterations in substrate oxidation, oxygen consumption, ATP synthesis, proton leak or electron transport will affect the RCR ratio and subsequently mitochondrial function in that tissue.

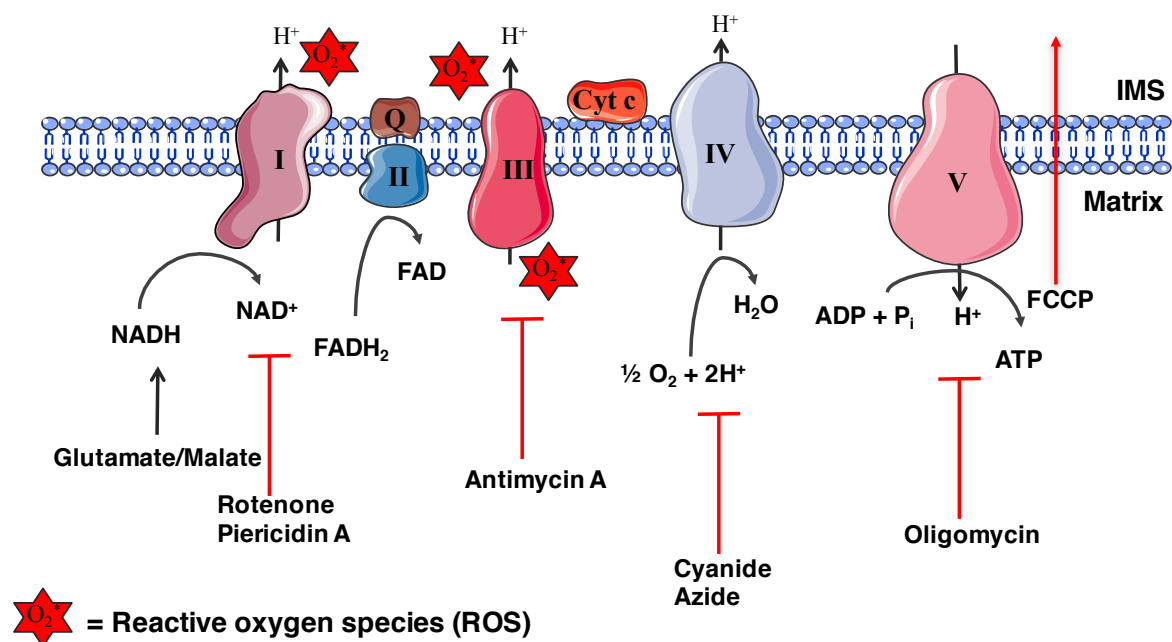


Figure. 1.2 - Selective inhibition of ETC/OXPHOS components. The electrochemical gradient can be dissipated by lipophilic protonophores carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) or 2,4-dinitrophenol (2,4-DNP) allowing diffusion of protons across the inner mitochondrial membrane. Electron transport can be inhibited at complex I with rotenone, piericidin A or 1-methyl-4-phenylpyridinium (MPP^+). Complex III can be inhibited with antimycin A and myxothiazol while azide and potassium cyanide inhibit Complex IV. Finally, ATP synthase can be inhibited by oligomycin.

Much can be learned from inhibiting the enzymes that make up the OXPHOS machinery and this can be done by a variety of chemical agents in a very specific manner. By inhibiting the ETC, two downstream effects are evoked; firstly, the rate of respiration is decreased as there are no electrons available to carry out the reduction of molecular oxygen and secondly, the F_1 portion of ATP synthase, turns in reverse, consuming ATP, and forces the F_0 portion to pump protons out

of the matrix in an attempt to maintain the PMF (Aksimentiev et al., 2004). ETC inhibitors generally stimulate oxidative stress by increasing the reduction level of carriers located upstream of the inhibition site. Complex I can be inhibited by compounds such as MPP⁺, rotenone or piericidin A as they block the transfer of electrons to CoQ. This has the effect of inhibiting respiration from NAD⁺ linked substrates and there are suggestions that these inhibitors also stimulate production of ROS by CI which can have deleterious effects on the cell, as will be discussed later (Turrens, 2003). Despite respiration via NADH being blocked, respiration through FAD linked substrates may still continue via complex II. Conversely, CII can also be inhibited by agents such as malonate and 3-nitropropionic acid (3-NP) whose mechanism of action also involves blocking the transfer of electrons from succinate dehydrogenase to CoQ (Binienda and Ali, 2001). Complex III can be inhibited with antimycin A or myxothizaol and complex IV can be inhibited with potassium cyanide (KCN) or sodium azide, both of which will completely block respiration from any substrate. Oligomycin inhibits ATP synthase by binding to and blocking rotation of the F₀ subunit thus preventing ATP production or consumption by reverse rotation resulting in inner membrane hyperpolarisation as no protons can re-enter the matrix despite being continually pumped out by the actions of the ETC. Eventually the $\Delta\Psi_m$ becomes so high there is insufficient energy to translocate protons against a steep concentration gradient and electron transport ceases (Zheng and Ramirez, 2000). Finally, the mitochondrial membrane potential can be completely dissipated by a class of compounds known as proton ionophores, such as FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazone) and 2,4-dinitrophenol (2,4-DNP), whose mechanism of inhibition involves transporting protons back across the inner membrane before their energy can be harnessed for ATP production, thus uncoupling the mitochondria. (Benz and McLaughlin, 1983).

As mentioned above, inhibition of some components of the ETC lead to the production of ROS, which is important because it underlies oxidative damage in many pathologies and contributes to redox signaling from the organelle to the cytosol and nucleus. Superoxide (O₂^{•-}) is the proximal mitochondrial ROS and its

production can lead to the subsequent generation of hydrogen peroxide (H_2O_2) and the hydroxyl radical ($-\text{OH}$) (Murphy, 2009). $\text{O}_2^{\bullet-}$ is produced within mitochondria by the one electron reduction of O_2 within complexes I and III, although some studies suggest that CIII only produces ROS when artificially inhibited (Adam-Vizi and Chinopoulos, 2006). CI production of ROS occurs by two mechanisms; when the matrix NADH/NAD^+ ratio is high, this leads to a reduced flavin mononucleotide site on CI, which will produce $\text{O}_2^{\bullet-}$ (Votyakova and Reynolds, 2001), and when electron supply reduces the CoQ pool in the presence of a high protonmotive force forcing the electrons back from CoQH_2 into CI, reducing NAD^+ to NADH at the flavin mononucleotide site, known as reverse electron transport (RET) (Chance and Hollunger, 1961). CIII production of ROS occurs when it is supplied with CoQH_2 and when the Q_i site is inhibited by antimycin A from the reaction of O_2 with a ubisemiquinone bound to the Q_o site (Zhang et al., 1998). However, in the absence of antimycin A, the Q_o site ubisemiquinone is not stabilized and $\text{O}_2^{\bullet-}$ production by CIII is low and so may not be physiologically relevant (Forman and Azzi, 1997). Accumulation of $\text{O}_2^{\bullet-}$ is associated with cell damage through oxidation of iron-sulfate clusters (Fridovich, 1997).

As mentioned, generation of ROS occurs under non-pathological conditions as around 1-2% of electrons escape the ETC (Dlasková et al., 2010). As a result, mitochondria have a mechanism to counter against the damaging effect of ROS production as non-enzymatic scavengers, such as CoQ and vitamin E (Ham and Liebler, 1995), and enzymatic scavengers including manganese superoxide dismutase (MnSOD). MnSOD is found in the mitochondrial matrix and catalyzes the conversion of $\text{O}_2^{\bullet-}$ to H_2O_2 , which is further neutralized by glutathione peroxidase or peroxiredoxins (de Moura and dos Santos, 2010). $\text{O}_2^{\bullet-}$ and $-\text{OH}$ are charged and cannot exit the matrix but H_2O_2 can freely diffuse across both mitochondrial membranes and enter the cytosol, where catalase is the most common neutralizing enzyme. This well-defined electron escape and ROS formation has a vital physiological signaling function (Radak et al., 2011) but it is when an imbalance between ROS production and antioxidant action occurs,

oxidative stress results and can seriously compromise cell integrity. The brain is a very sensitive organ to oxidative stress due to its high metabolic rate and the large amount of iron and copper found there, which interact with ROS producing the highly reactive hydroxyl radical that yields damage to proteins, lipids and DNA and over time accumulations of this damage could have a causative role in the onset of several neurodegenerative disorders. Mitochondria are highly efficient organelles that are essential for cellular metabolism and energy production but this process of ATP production must be tightly controlled so as not to initiate a destructive cascade of cellular damage, highlighting the importance of mitochondrial dynamics, as discussed below.

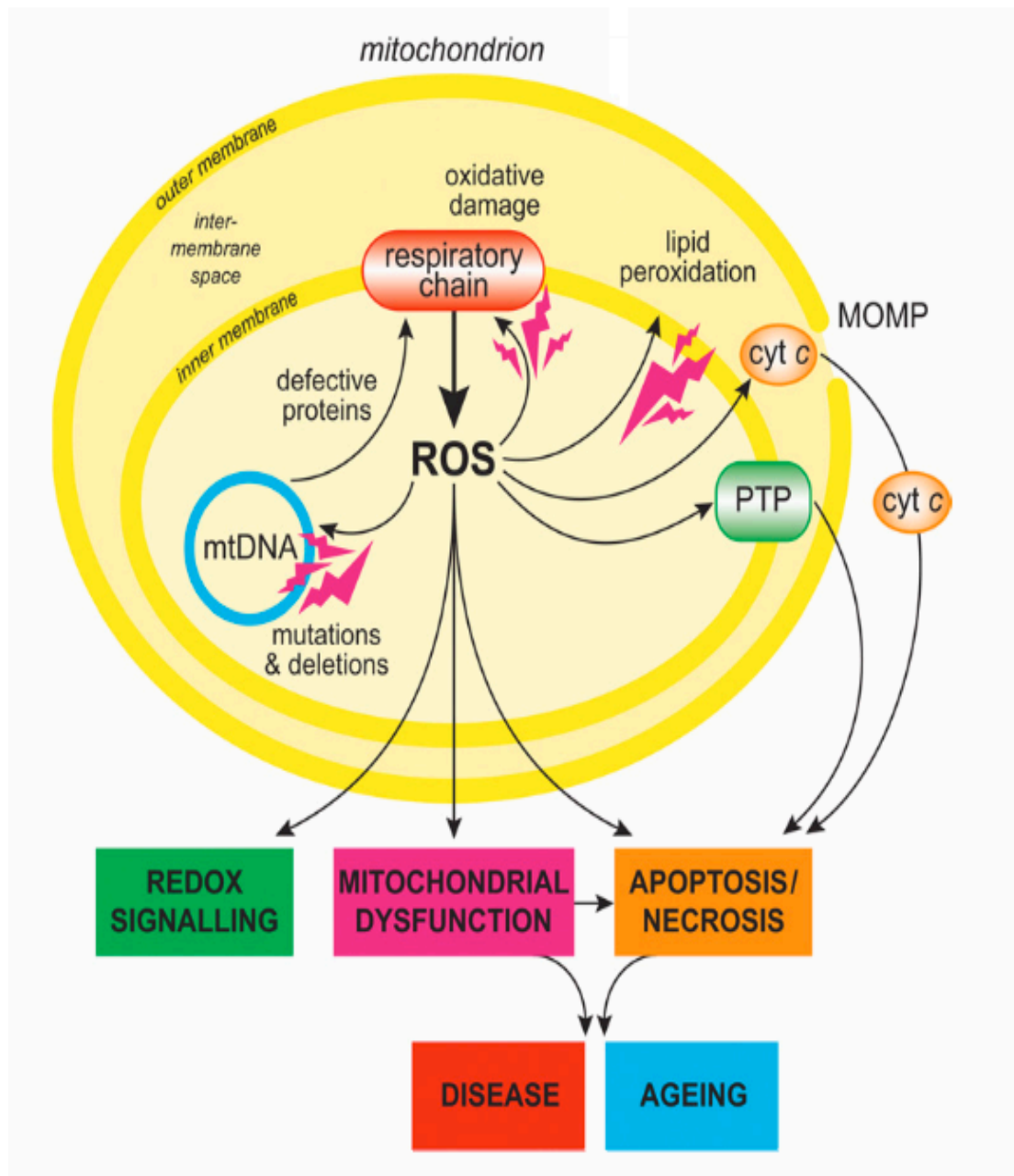


Figure 1.3 – Production of Reactive Oxygen Species (ROS) occurs within mitochondria. ROS production can lead to oxidative damage to mitochondrial proteins, membranes and DNA resulting in a wide variety of dysfunctions within the organelle including deficits in ATP productions, dynamics and redox signaling. Taken from Murphy (2009).

1.3 Mitochondrial Dynamics

Until recently, there was a common preconception in the scientific world that mitochondria were static organelles composed of a double membrane with infolds called cristae that provided energy to their local environment. However, with major developments in the past 25 years we now know that mitochondria are highly dynamic organelles that undergo constant cycles of fission and fusion to maintain normal cellular function. This is known as mitochondrial dynamics and refers not only to the processes of fission and fusion but also to the regulated movement, morphological changes and elimination of damaged mitochondria as part of quality control (Roy et al., 2015). The processes of fusion and fission have been conserved from yeast up to mammals and are controlled by four large GTPases; mitofusins Mfn1 and Mfn2 (Fzo1 in yeast) for outer membrane fusion, Opa1 (Mgm1 in yeast) for inner membrane fusion and Drp1 (Dnm1 in yeast) for the fission process (Otera and Mihara, 2011). Recent evidence has also shown more minor roles for a variety of other proteins in the processes of fusion and fission including dynamin-1 (Dyn1) and dynamin-2 (Dyn2) in the completion of the fission process (Lee et al., 2016). Movement of mitochondria is facilitated along a cytoskeletal network of microtubules where a kinesin motor directs them in the anterograde direction and a dynein motor directs them in the retrograde direction (Ni et al., 2015). The attachment of mitochondria to these motor proteins is regulated by a series of adapter proteins such as Milton, which links to kinesin via the outer membrane protein Mitochondrial Rho GTPase (Miro) while Mfn1 and Mfn2 also interact with Miro and Milton (Saxton and Hollenbeck, 2012).

The movement of mitochondria is not continuous but instead saltatory with stops followed by a change in direction, potentially reflecting the attachment or detachment of kinesin and dynein motor proteins (Detmer and Chan, 2007). This movement is perhaps best studied in neurons where it has been shown that this is not a slow process, with speeds of movement in the anterograde or retrograde direction along axons having been recorded at speeds of up to $1\mu\text{m sec}^{-1}$ (Pilling et al., 2006, Morris and Hollenbeck, 1995). There are also several lines of

evidence that suggest that this mitochondrial movement within neurons is highly regulated. Firstly, it has been shown that mitochondria accumulate at regions of local stimulation with Nerve Growth Factor (NGF) in sensory neurons and that these local high NGF concentrations inhibit movement of mitochondria out of the region (Chada and Hollenbeck, 2004). Secondly, mitochondria with a higher $\Delta\Psi_m$ are transported in the anterograde direction towards the growth cone or nerve terminal while those with a lower $\Delta\Psi_m$ are transported in the retrograde direction back towards the cell soma (Miller and Sheetz, 2004). Thirdly, as is shown in the same paper, mitochondria move towards areas with a low population of healthy mitochondria. As well as this, mitochondria are trafficked towards regions of the cell with the highest energy demands, such as post-synaptic densities and pre-synaptic sites (Detmer and Chan, 2007). Finally, mitochondria are extremely sensitive to Ca^{2+} fluctuations, even playing a role in intracellular Ca^{2+} signaling, which also links transport to pre-synaptic sites as high levels of Ca^{2+} are required for synaptic vesicle release (Rizzuto et al., 2012). This highly dynamic and regulated movement of mitochondria ensures proper inheritance of mitochondria and their vital DNA and lipid components as well as maintaining overall cellular function.

Another aspect of mitochondrial dynamics is the ability to maintain a healthy mitochondrial population in order to keep the cells energy capacity at a high level and also to avoid activation of the apoptotic pathway as mitochondria are central executioners of this pathway. The main way in which mitochondria do this is by the processes of fission and fusion whereby healthy components are shared and damaged components can be segregated for degradation (Gilad et al., 2008), which will be discussed further below, but there are several other mechanisms by which mitochondria carry out quality control. These organelles have been shown to have their own proteolytic system which assists in the degradation of mis-folded proteins that could interfere with mitochondrial function (Brooke and Cole, 2011). Under oxidative stress conditions or in areas of high ROS production, mitochondria become rounded buds that break off to form mitochondria-derived vesicles (MDV), which then fuse with lysosomes where oxidized proteins are

degraded (Vincent et al., 2012). When damage has progressed significantly and “budding off” will no longer degrade all damaged proteins, whole mitochondria can acquire lysosomal markers thus preventing the inheritance of damaged proteins (Wen-Xing et al., 2012). This likely serves as an alternative pathway when damage has proceeded to an extent that the fission process will not remove all oxidized proteins. Finally, the fission process itself appears to have a vital role in mitochondrial quality control. After fission, daughter mitochondria have either an increased or decreased membrane potential, the one with the higher membrane potential proceeds to future fusion events while the one with lower membrane potential is degraded by mitophagy (Ni et al., 2015). This bears resonance with the observation of Miller and Sheetz (2004) that mitochondria with higher membrane potential are transported to the nerve terminal but those with lower membrane potential move in the retrograde direction towards the cell soma suggesting that degradation occurs here in the neuron.

1.3.1 Mitochondrial Transport

Mitochondrial transport of neurons is orchestrated by connection to microtubule filaments by plus end directed kinesin-1/KIF5 and minus end directed cytoplasmic dynein. The myriad of factors that control mitochondrial motility, as detailed above, suggest that mitochondria move in an anterograde or retrograde direction based on connection to motor assemblies as opposed to individual motors at any given time as evidenced by isolation of mitochondria that have up to 100 kinesin molecules attached from brain tissue (Leopold et al., 1992). The direction of movement of the mitochondrion depends on interplay between 3 distinct factors; microtubule orientation, the number of attached kinesin and dynein molecules and the extent to which the motors are actively driving transport.

Microtubule stability and orientation varies extensively in neurons between axons and dendrites and also between cell types which has important implications for the mechanisms required to balance anterograde and retrograde transport. In axons, microtubule arrays almost always have their plus end orientated out while in dendrites the orientation is more heterogenous, depending on distance from

the soma (Baas et al., 1988). Thus in axons, kinesin driven anterograde transport must be balanced with retrograde driven dynein transport but in dendrites with mixed orientation microtubules, either dynein or kinesin could drive both anterograde and retrograde transport (Barnhart, 2016). The interaction between mitochondria and microtubules is mediated by adaptor complexes, the best studied of which is the Miro-Milton complex. Miro is expressed on the mitochondrial outer membrane and binds to Milton, which then binds to microtubule motors (Glater et al., 2006). There are 2 mammalian homologs of Milton called TRAK1 and TRAK2 which bind to dynein and kinesin. TRAK1 localizes most commonly to axons while TRAK2 localizes to dendrites and primarily binds to dynein meaning that transport of mitochondria within dendrites is reliant on TRAK2 binding to microtubules with opposites orientations (van Spronsen et al., 2013). Finally, microtubule-motor engagement is influenced by a variety of factors including interactions with binding partners, such as LIS1, and post translational modifications of motors and microtubules (McKenney et al., 2010, Gibbs et al., 2015). For example, increased O-GlcNAcylation of Milton (TRAK1/2) results in decreased mitochondrial motility but this will be further investigated in a later section (Pekkurnaz et al., 2014).

1.3.2 Mitochondrial Fusion

Fusion between closely apposed mitochondria is a complex process that involves the fusing of two membranes and sharing of mitochondrial contents and so requires the input of several proteins. The first of these, *fuzzy onions* (fzo), was discovered in *Drosophila Melongaster* and was found to be essential for spermatogenesis in the fruitfly as this process requires the fusion and elongation of mitochondria in postmeiotic spermatids. Fzo mutant males are defective in this developmentally regulated mitochondrial fusion and are sterile (Hales and Fuller, 1997). Subsequent research led to the discovery of its yeast (Fzo1) and mammalian homologs (Mfn1 and Mfn2). These 2 large GTPase proteins were found to have 77% similarity to each other with highest homology in the GTPase domain and least conserved in C terminal region. They were also found to have overlapping but not completely redundant functions in MOM fusion but knockout

of either leads to prenatal death (Santel and Fuller, 2001, Chen et al., 2003). Both are anchored to the MOM with an N-terminal GTPase domain while the C-terminal domain is a coiled-coil exposed to the cytosol and MOM fusion is GTPase dependent. Both proteins form homo- or hetero-protein complexes and this protein oligomerization is mediated by a heptad repeat region (HR2) (Koshiba et al., 2004). These two proteins have varying expression patterns in mammalian tissue, likely relating to the rate of mitochondrial fusion in particular tissues or potential functions independent of fusion. Mfn2 is highly expressed in cardiac tissue and skeletal muscle in accordance with its role in Ca^{2+} signaling during mitochondrial-ER tethering, while Mfn1 has been identified as enhancing virus-responsive reporter activity suggesting a potential association with increased susceptibility to certain pathogen infections (de Brito and Scorrano, 2008) (Yoneyama et al., 2004).

The mechanism of fusion in mitochondria is still poorly understood and research is ongoing to determine the exact the method but it is believed to involve GTP hydrolysis. The best-studied model of membrane fusion is in synaptic vesicle fusion with the neuronal outer membrane that utilizes a SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex. Firstly, the vesicle becomes 'tethered' to the target membrane although a gap remains between them. This step is mediated by a group of GTPases on the vesicle membrane known as Rab guanosine triphosphatases. Next, SNARE proteins on both membranes form a complex leading to the membranes attaining a closer proximity in a state termed 'docking'. Finally, the two bilayers fuse owing to the close proximity brought about by the SNARE complex formation. Interestingly, the defining feature of SNARE proteins is an extended coiled-coil stretch as is seen in the C-terminal of the mitofusins (Jahn and Fasshauer, 2012). It is believed that Mfn1 and 2 act as SNARE proteins and research published by Koshiba et al. (2004) provided us with a detailed mechanism of how MOM fusion is carried out. The *trans* homo-and hetero-oligomeric complexes formed by Mfn1/2 tether apposing membranes together with a distance of around 95Å separating them. This 'tethering' is believed to be mediated by the HR2 regions of either protein as

when they are isolated from the total protein, homo- and heterotypic complexes are formed. Mfn1 appears to be particularly important in this tethering stage as they showed that cells expressing a mutant form of Mfn1 without any GTPase activity showed a mitochondrial phenotype in which they were aggregated and trapped in a “tethered” state. Coupled to this, exogenous expression of wild type Mfn1 can rescue cells expressing Mfn2 disease mutants but expression of wild type Mfn2 cannot (Detmer and Chan, 2007). Further evidence for an interaction between both mitofusins was provided when it was shown that a heterotypic Mfn1-Mfn2 *trans* complex had greater efficacy in fusion events than homotypic Mfn1 or Mfn2 complexes (Hoppins et al., 2011). Furthermore, binding of HR1 and HR2 in homotypic Mfn2 complexes have been shown to be inhibitory to MOM fusion (Huang et al., 2011). A negative regulator of Mfn1/2 has also been identified in mitofusin binding protein (MIB). MIB is a member of the medium chain dehydrogenase/reductase protein superfamily and overexpression in HeLa cells results in mitochondrial fragmentation while gene silencing results in an elongated mitochondrial morphology (Eura et al., 2006). Despite these advances in modeling and comparisons to SNARE vesicle fusion, the mechanistic details of fusion that follow GTP hydrolysis have yet to be elucidated.

The process of fusion between two mitochondria is quick and is completed in less than 2 minutes, however it can occur in the absence of any nuclear instruction or *de novo* translation which suggests it's an autonomous process occurring in response to cellular cues (Schauss et al., 2010). It should be noted that despite the similarities, SNARE proteins range in size from 80 to 100 amino acids while Mfn1 and 2 are 741 and 757 amino acids long respectively indicating their role as multifunctional receptors with additional roles independent of fusion. The proposed major function of mitochondrial fusion is the sharing of mitochondrial contents to maintain heterogeneity yet this appears to be an insignificant function for a process that is vital for organismal survival (Chen et al., 2003, Davies et al., 2007). Instead it seems more likely that the key role of mitochondrial fusion is to marry the bioenergetic state of the cell with mitochondrial function, and some of the ways in which it does this is through sharing of mtDNA content and alteration

of mitochondrial morphology. This idea is supported by the fact that fusion can be inhibited or activated by the cytosolic milieu including phosphorylation events, oxidized glutathione or other post translational modification including O-GlcNAcylation (Pyakurel et al., 2015, Shutt et al., 2012). The other protein involved in mitochondrial fusion, Opa1, supports this hypothesis as it acts as a cellular stress sensor in order to modulate mitochondrial function.

Opa1, or Optic Atrophy type 1, is a large GTPase required for inner mitochondrial membrane fusion, so called because mutations in the gene are the most common cause of this dominantly inherited optic neuropathy that leads to progressive loss in visual acuity and eventually blindness (Delettre et al., 2000). It is a dynamin-like protein with a sequence homology to the yeast gene Mgm1, responsible for maintaining mtDNA and inner membrane structures in yeast while also interacting with Fzo1 to regulate mitochondrial fusion. Unlike Mfn1/2, Opa1 is only required on one MIM for mitochondrial fusion to occur (Song et al., 2009a). Exogenous expression of OPA1 in mammalian cell lines results in an altered mitochondrial morphology while knockdown using siRNA results in fragmented mitochondria proving it's involvement in MIM fusion (Ishihara et al., 2006). Opa1 is targeted to mitochondria via a basic-rich N-terminal targeting sequence where it is anchored within the MIM facing the intra membrane space (Olichon et al., 2002). Opa1 functions are controlled by a complex pattern of alternative splicing and proteolysis. There are 8 different isoforms which all have the mitochondrial leader sequence mentioned above. This is cleaved by the mitochondrial processing peptidase (MPP) upon import into the mitochondria (Head et al., 2009). From here, Opa1 processing follows two distinct directions. If the isoform contains alternatively spliced exons 4b or 5b, they are constitutively cleaved by the intra-membrane space AAA protease YME1L1 which generates the short form of Opa1 (S-Opa1) while the remaining isoforms are not cleaved further generating the long form (L-Opa1). This process is regulated by a group of MIM proteins known as prohibitins (Otera and Mihara, 2011, Griparic et al., 2007). The shorter isoforms are more loosely bound to the IM than the long forms that retain their hydrophobic domain and it appears that this may have a functional significance (Ishihara et al.,

2006). Loss of $\Delta\Psi_m$ results in a significant fragmentation of mitochondria which is closely linked to a rapid conversion of L-Opa1 to S-Opa1 mediated by Oma1, a protease with multiple membrane spanning segments and a zinc binding motif (McBride and Soubannier, 2010).

It is unclear if MIM and MOM fusion occur simultaneously or sequentially or completely separately. The two processes can be separated by dissipation of the inner membrane potential with valinomycin or CCCP as shown by Malka et al. (2005) but they also demonstrated that they proceed separately in the absence of any pharmacological interference suggesting they can function separately. This was further backed up by evidence that MOM fusion can proceed in OPA1 null MEFs and that OPA1 is not required on adjacent mitochondria for MIM fusion as OPA1-null/wildtype cell hybrids exhibited mostly partial mitochondrial fusion (Song et al., 2009b). Finally MIM fusion appears to be Mfn-1 dependent (Guillery et al., 2008) so we can conclude that MOM fusion can proceed without MIM fusion but the latter requires the presence of Mfn-1. Increased OXPHOS activity and higher levels of ATP are associated with elongated mitochondria as this type of mitochondrion appears to have more efficient energy generation and is capable of distributing energy over long distances (Mittra et al., 2009, Skulachev, 2001). This likely occurs as increased OXPHOS activity can then stimulate inner membrane fusion while outer membrane fusion remains unaffected (Mishra et al., 2014) further highlighting the separation of the processes and the role of a metabolic sensor via its alternative proteolysis by Yme1L or Oma1. Opa1 has further roles in cristae remodeling and supercomplex formation indicating the importance of other roles for these fusion proteins in cellular metabolism and how closely linked mitochondrial bioenergetics and dynamics are (Cogliati et al., 2013).

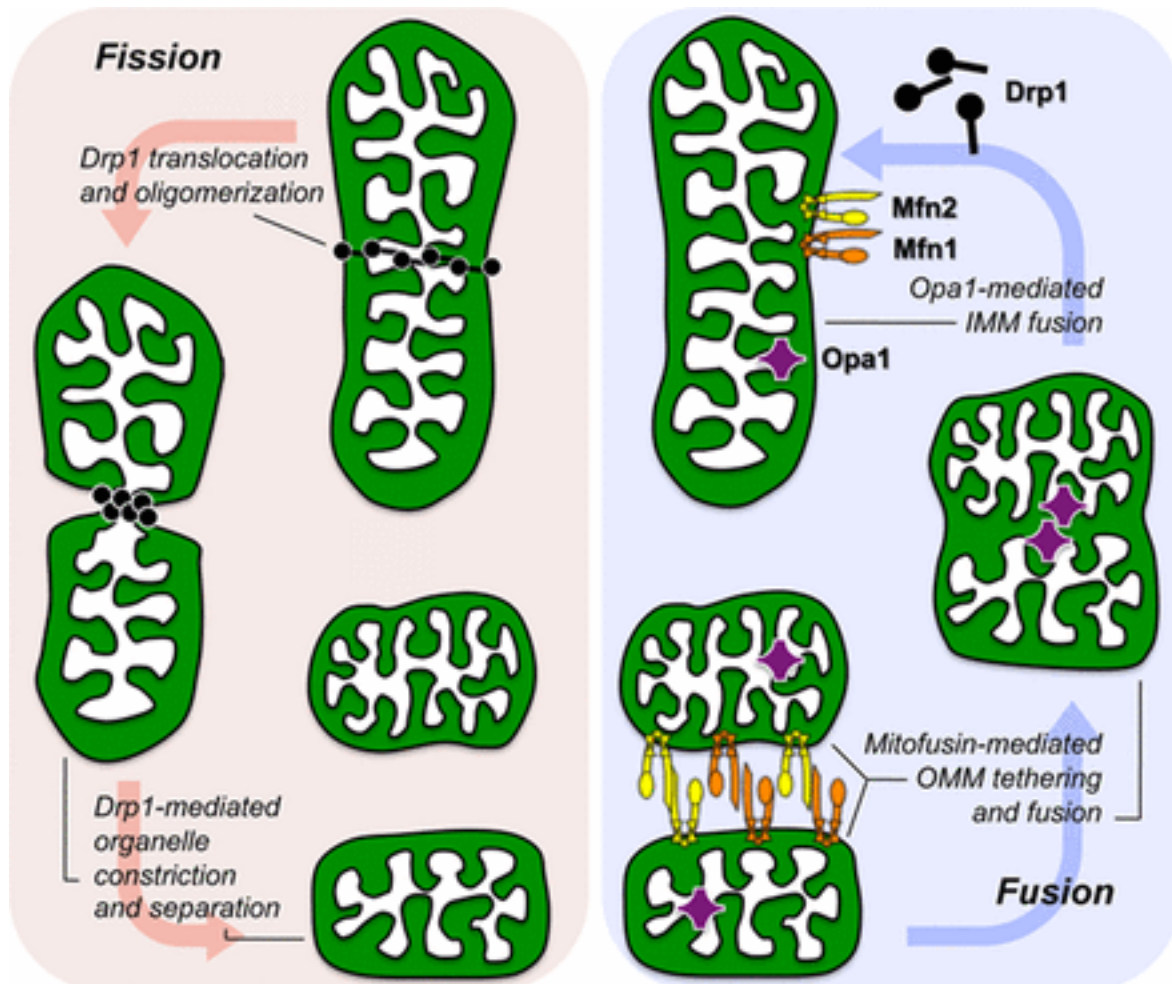


Figure 1.4 Putative Mechanisms of mitochondrial fusion and fission. During fission, Dynamin related protein 1 (Drp1) is dephosphorylated by calcineurin and recruited to the outer mitochondrial membrane. Once recruited, Drp1 forms an oligomeric ring around the outer membrane with the aid of the microtubule cytoskeleton. Upon GTP hydrolysis, this ring structure constricts causing membrane scission and fission of the mitochondrion. During fusion, Mitofusins 1 and 2 (Mfn1/2) assemble in homo- and hetero oligomers and mediate fusion via apposition of mitochondria in *trans*. Optic Atrophy 1 (OPA1) is localized to the inner mitochondrial membrane and plays an important role in inner membrane fusion although the exact mechanism of action is currently unclear. Adapted from Dorn and Kitsis (2015).

1.3.2 Mitochondrial Fission

As with mitochondrial fusion, model organisms have provided us with vital information in the machinery provided for mitochondrial fission through the identification of the gene Dnm1p in yeast which was followed by the mammalian orthologue dynamin related protein 1 (Drp1). Drp1 is a cytosolic, microtubule associated protein containing a GTPase domain, a putative pleckstrin homology (PH)-like domain and a GTPase effector domain (Kageyama et al., 2011). Loss of Drp1 function results in long, interconnected mitochondrial networks reflective of an impairment in mitochondrial fission (Roy et al., 2015). As it is a cytosolic protein, recruitment of Drp1 to the MOM requires a number of receptors. The first of these, Fis1, was also discovered in yeast and was shown to be required for the proper assembly and distribution of Dnm1p containing fission complexes on mitochondrial tubules (Mozdy et al., 2000). Despite the finding of a mammalian homolog, further research in mammalian cells suggested that the interaction between Fis1 and Drp1 has a minor role in regulating mitochondrial fission as mitochondrial recruitment of Drp1 is not affected by Fis1 knockdown (Lee et al., 2004). Instead, it is likely that the recruitment of Drp1 to the MOM requires other receptors such as a mitochondrial fission factor (Mff) and Mid49/51. Mff is tail anchored in the MOM, existing in a 200kDa complex and knockdown has been shown to inhibit fission induced by loss of mitochondrial membrane potential (Gandre-Babbe and van der Bliek, 2008) and release the Drp1 foci from the MOM while overexpression results in the mitochondrial recruitment of Drp1 and increased fission (Otera et al., 2010). Mid49/51 were discovered as a result of a mitochondrial proteomic screen and it is thought that Mid51 stimulates the GTPase activity of Drp1 in the presence of ADP implying it also acts as a metabolic sensor and regulator of fission (Losón et al., 2014).

The precise mechanism of action of Drp1 once it is recruited to the MOM is not yet known but there are a number of post translational modifications that effect its function. Phosphorylation by Cdk1/cyclin B at Ser616 increases GTPase activity but dephosphorylation by phosphatase calcineurin at Ser637 leads to mitochondrial elongation (Ni et al., 2015). Drp1 can also be ubiquitinated by

MARCH5 or sumoylated by SUMO-1 which can either regulate the stability of Drp1 or recruit Drp1 to the actual division site respectively (Karbowsky et al., 2007). The fission process occurs by Drp1 assembling into higher order structures that wrap around the mitochondrial tubule, constricting and eventually severing the mitochondrial membrane by a GTP hydrolysis-dependent mechanism (Ingeman et al., 2005). It is not known if the fission of the MIM and MOM are separate events but it is plausible that the constriction of the Drp1 oligomeric ring is sufficient to sever both membranes. At present the only identified MIM protein with a role in fusion is MTP18 which is a transcriptionally regulated target of phosphatidylinositol 3-kinase (IP3 kinase) signaling and regulates mitochondrial fission coupled with the action of Drp1 so its precise role in MIM fission is unknown (Tondera et al., 2005). It is postulated that the ER may also be involved in the fission process as ER tubules appear to encircle and constrict mitochondrial tubules prior to the recruitment of Drp1 while Drp1 receptors are also located at the ER-mitochondria contact site (Friedman et al., 2011). Recent research has demonstrated that actin polymerization at the ER-mitochondria contact site (MAM-mitochondrion associated membrane) through the ER-localized formin 2 (INF2) protein and the recruitment of myosin II allow for efficient fission by facilitating Drp1 assembly at this site (Korobova et al., 2014, Korobova et al., 2013).

Drp1 also has multiple ways of sensing its metabolic microenvironment and adjusting mitochondrial function accordingly. The best studied of these is phosphorylation which can occur at a number of sites on the protein including two critical sites serine 616 and serine 637. Protein kinase A (PKA) phosphorylates Drp1 at S637 which inhibits its activity resulting in an elongated mitochondrial phenotype as a result of pharmacological activation, β -adrenergic stimulation (Cribbs and Strack, 2007) or mTOR inhibition which increases cAMP levels and subsequently activates PKA (Gomes et al., 2011b). On the contrary, dephosphorylation at S637 by the Ca^{2+} dependent phosphatase calcineurin results in a promotion of Drp1 activity and recruitment to the mitochondrial surface (Cereghetti and Stangherlin, 2008). This is relevant to metabolic activity as

dysfunction of the calcium buffering capacity of mitochondria would give rise to an increase in cytosolic Ca^{2+} and trigger calcineurin dependent Drp1 activation and mitochondrial fission. Another link between bioenergetics and mitochondrial fission is evident when respiration is inhibited and excess ADP binds MiD51 stimulating Drp1 spiral assembly, GTP hydrolysis and thus promoting fission (Pernas and Scorrano, 2016). Additionally AMP kinase activation results in a phosphorylation of Mff and so promotes mitochondrial fission linking energy deficiency to mitochondrial fragmentation (Toyama et al., 2016).

Thus, the dynamic nature of mitochondria, although not governed by any *de novo* translation, is affected by a series of intracellular sensors which can detect subtle changes in membrane potential, Ca^{2+} concentration and metabolism in order to alter the morphology and activity of the mitochondria through a series of post-translational modifications. The fusion/fission machinery link the metabolic state of the entire cell to mitochondrial function ensuring optimum activity depending on cell type and substrate availability.

1.4 Mitochondria and Neurodegeneration

Neurons have a unique morphology in that their cell soma which contains the nucleus and much of the required machinery for protein synthesis as well as many other organelles vital for normal cellular functions can be located up to a meter away from the site of main neuronal activity, the synapse (Knott et al., 2008). This places extensive demands on the cell as it involves the trafficking of proteins, nucleic acids and even organelles to the site of neurotransmission. Neurotransmission itself is an energetically expensive process, as it requires the production of neurotransmitters and specialized vesicles in which to store them, the ability to depolarize the plasma membrane through opening of ion pores, Ca^{2+} release to stimulate neurotransmitter release and a variety of other functions in order to maintain normal neuronal homeostasis. This places extreme energy demands on neurons, explaining why they are the most energetically active cells in the body (Millet and Gillette, 2012), while the fact that they derive over 90% of required ATP through OXPHOS puts additional pressure on normal mitochondrial function (Cai and Sheng, 2009). The increased pressure on mitochondria is extremely relevant to how mitochondrial dynamics adapt to this, in particular the active transport along microtubules to areas of high energy demand which can be vast distances away.

It is perhaps unsurprising then, that dysfunction in mitochondria has been linked to the onset and/or progression of a wide array of adult neurodegenerative disorders including Alzheimer's Disease (AD), Parkinson's Disease (PD) and Huntington's Disease (HD) amongst others. These diseases encompass a larger group of conditions characterized by the selective death of neuronal subtypes known as adult onset neurodegenerative disorders. In general, they arise sporadically, although there is a genetic component to certain forms of each disease, and the most common risk factor appears to be age as numbers of cases diagnosed increase exponentially from age 60 onwards. Despite the wildly varying phenotype of the various conditions there appear to be some common underlying themes including ER stress, protein mis-folding and aggregation,

oxidative stress, neuro-inflammation and impaired mitochondrial function (Schon and Przedborski, 2011). This mitochondrial dysfunction is of particular interest as it relates not only to metabolic breakdown but also to dysfunctioning mitochondrial dynamics and/or the link between the two. The following sections outline the current knowledge of mitochondrial dysfunction in a variety of the most common neurodegenerative disorders.

1.4.1 Mitochondrial Dysfunction and Alzheimer's Disease

AD is an age related, progressive, adult onset neurodegenerative disorder characterized by selective neuronal death in the cerebral cortex (especially noteworthy in the hippocampus, parts of the frontal lobes and the cingulate gyrus), cognitive dysfunction and two pathological hallmarks; senile plaques formed by extracellular deposits of amyloid- β ($A\beta$) peptides and neurofibrillary tangles (NFT) composed of intracellular aggregations of hyperphosphorylated tau protein (Dominic and Dennis, 2007). The presence of these protein aggregates appears to lead to neuronal cell death but the exact mechanisms by which the apoptotic pathway is activated has yet to be elucidated. $A\beta$ in particular provides many major pieces of evidence in support of a causative role in AD such as duplication or mutation of the amyloid precursor protein (APP) (Rovelet-Lecrux et al., 2006) and the observance of the toxicity of $A\beta$ peptides to hippocampal neurons in culture and *in vivo* (Deshpande et al., 2006). Depending on the exact point of cleavage of APP by γ -secretase, three principal forms of $A\beta$ are produced, comprising of 38,40 or 42 amino acid residues. Of these, it appears that $A\beta_{42}$ is the most likely to oligomerize and potentiate neuronal death (Burdick et al., 1992). Despite all this evidence, the mechanism of toxicity of $A\beta$ has not been shown and there is a large body of evidence showing metabolic breakdown in the brains of AD patients and $A\beta$ has even been shown to selectively inhibit complex IV in rat brain in a dose dependent manner (Casley et al., 2002). In addition, there is strong evidence of excessive ROS production in AD brains, alterations in the MAM, mitochondrial dynamics and post-translational modifications of Drp1 as discussed below.

The presence of A β in extracellular plaques has been well documented but it has also been seen to localize to several cellular organelles including the ER, the Golgi apparatus and the mitochondrion (Picone et al., 2014). The mitochondrion is of particular interest as A β has been found to accumulate in the mitochondria of the brains of AD patients and transgenic mice (Caspersen et al., 2005). It has been shown by Petersen et al. (2008) that this protein is not synthesized within the mitochondrion but is derived from its intracellular or extracellular pool and is transported to the mitochondrion via a specific mechanism involving the translocase of the outer membrane (TOM) complex. This was supplemented with further data from confocal microscopy showing the colocalization of A β with complex II, the MIM and the mitochondrial chaperon protein Hsp60 (Walls et al., 2012).

As mentioned previously, imbalance of ROS production induces irreversible damage to a wide variety of cellular components inducing the apoptotic pathway. It has been shown in neuroblastoma LAN5 cells that A β is a key factor in free radical generation, oxidative damage and mitochondrial dysfunction (Marta Di et al., 2012). More recently, an eloquent imaging study by Xie et al. (2013) showed pathological changes in the brains of transgenic animals developing amyloid deposits. In brain regions close to developing A β plaques, there were a decreased number of mitochondria, many of which were fragmented and showing a reduced membrane potential. There are also some results suggesting oxidative stress may activate signaling pathways that alter APP processing as β -secretase expression is increased under oxidative stress through activation of c-Jun amino-terminal kinase and p38 mitogen-activated protein kinase (MAPK) (Elena et al., 2005). There is also evidence linking the mitochondrial-ER contact site, the MAM, to increased function following oxidative stress meaning that this could be a downstream effect of increased A β aggregation in mitochondria. MAM function is increased in model systems of familial and sporadic AD and an increased expression of MAM associated proteins has been detected in post mortem AD brains (Hedskog et al., 2013).

Mitochondrial dynamics have also been implicated in AD as shown by Wang et al. (2008) when they demonstrated that overexpression of APP resulted in ~ 40% of M17 cells displaying alterations in mitochondrial morphology. These results were supplemented with data showing the overexpression of a mutant form of APP associated with AD resulted in < 80% of cells displaying the same fragmented structure and abnormal distribution around the perinuclear area suggesting an increase in fission and/or disrupted fusion events. This effect has also been seen in human AD patients where mitochondrial morphological changes manifested as fragmented mitochondria with damaged inner membrane structures have been reported in neurons (Wang et al., 2009). Drp1 interactions with A β and phosphorylated tau have been identified in post mortem brains of AD patients and it is hypothesized that this interaction is responsible for the increased mitochondrial fission and decreased fusion also observed in the brains of both post mortem patients and mouse models (Manczak and Reddy, 2012). A possible intermediate in this pathway is nitric oxide (NO), as it is produced in response to A β , which nitrosylates Drp1 triggering mitochondrial fragmentation (Cho et al., 2009). In support of this interaction, inhibition of mitochondrial fragmentation by partial Drp1 deficiency has been shown to be sufficient to alleviate mitochondrial dysfunction and synaptic loss in both the APP and tau transgenic mouse models of AD while also decreasing production of phosphorylated tau (Kandimalla et al., 2016, Manczak et al., 2016). There are a wide variety of abnormalities mentioned above suggesting the most likely hypothesis for mitochondrial dysfunction in AD is that an accumulation of A β in the mitochondrion triggers a cascade that results in a gradual breakdown of mitochondrial activity and dynamics, which may result in the activation of the apoptotic pathway leading to neuronal cell death.

1.4.2 Mitochondrial Dysfunction and Parkinson's Disease

Parkinson's Disease (PD) is another adult onset, progressive, neurodegenerative disease, characterized by loss of dopaminergic (DA) neurons in the substantia nigra (SN) and associated with α -synuclein protein aggregates, known as Lewy bodies. The manifestation of this loss of DA neurons is known as parkinsonism

and includes a tremor, increased muscle tone, slow movement and impaired gait (Spillantini and Goedert, 2013). Despite the α -synuclein aggregation not affecting the motor cortex, movement deficits are observed because the neurons of the SN project to many other basal ganglia nuclei, which have essential roles in the initiation of movement and also explain the other non-motor symptoms associated this PD such as sleep disturbances, pain and neuropsychiatric symptoms (Chaudhuri and Schapira, 2009). As with AD, the primary cause of PD has yet to be identified despite widespread study of the pathophysiology symptomatic expression of the disease. This has led to the recognition that many cellular processes are interrupted as the disease progresses including oxidative stress, the ubiquitin-protease system, mitochondrial dysfunction and excitotoxicity (Haelterman et al., 2014). At present, mitochondrial dysfunction seems like the most likely candidate for the primary cause of PD based on numerous studies indicating a deficiency of complex I activity in the SN of PD patients (Keeney et al., 2006) and observations that drug users who took opioid analogues (MPPP) laced with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) developed PD like symptoms and showed degeneration of DA neurons in the SN. In the body, MPTP is converted into MPP^+ which crosses the blood brain barrier and is selectively imported into DA neurons, inhibiting CI (Haelterman et al., 2014).

Over the last number of years genes have been identified which are responsible for early onset PD or contain mutations which increase susceptibility for late onset PD including pink1, parkin, LRRK2 and α - synuclein itself (Singleton et al., 2003). Pink1 and Parkin play important roles in regulating mitochondrial morphology and are involved in mitochondrial quality control by inducing clearance of dysfunctional mitochondria. Under basal conditions, low levels of the kinase Pink1 span both the MIM and the MOM where it acts on respirasomes and regulates CI activity. Knockout of Pink1 leads to a reduction in CI activity in flies, mice and humans (Morais et al., 2009) but it has recently been suggested that Pink1 regulates the protein turnover of multiple subunits of all four ETC complexes and ATP synthase (Vincow et al., 2013). Parkin is an E3 ubiquitin ligase, normally present in the cytosol and only recruited to mitochondria when they are

dysfunctional. Parkin mutants do not display CI defects but they do show reduced ATP levels in the fruit fly, similarly to Pink1 mutants (Vos et al., 2012). This effect seems to be related to the post-translational modification activity of parkin whereby it ubiquitinates and thereby regulates protein levels of many of the subunits of ETC complexes (Sarraf et al., 2013). Together, these 2 proteins appear to be required for ATP production, although both proteins affect the activity of different complexes with the ETC while their role in mitophagy may also prove to be relevant to the disease progression of PD.

The main pathological hallmark of PD is an accumulation of α -synuclein in the cytoplasm and, as has been discovered more recently, in the mitochondria. Devi et al. (2008) showed that human α -synuclein has a cryptic mitochondrial targeting sequence in its N-terminal and that it predominantly associates with the MIM. Further to that, they also demonstrated that accumulation of α -synuclein in the mitochondria of human dopaminergic neurons caused a reduction in CI activity and increased production of ROS and they highlighted that these effects occur at an earlier time point in dopaminergic neurons expressing α -synuclein with an A53T mutation associated with familial PD. It has also been shown that elevated ROS levels may enhance the mitochondrial localization of α -synuclein (Cole et al., 2008) thus commencing a vicious cycle whereby misfolded or accumulated protein inhibits CI, increases ROS and potentiates its own mitochondrial localization.

There is much evidence linking an impairment in mitochondrial dynamics to PD. In *Drosophila*, mutations in Pink1 or Parkin result in mitochondria that are swollen and lose their typical shape but these morphological defects can be overcome by decreasing fusion or increasing fission in both mutant forms (Poole et al., 2008). There is also a role for Pink1 and Parkin in mitochondrial trafficking as Pink1 phosphorylates Miro, triggering its degradation via a Parkin dependent ubiquitination pathway causing mitochondria to detach from kinesin motors thus reducing mitochondrial motility (Liu et al., 2012a). Finally, many groups have reported that overexpression of the mutant form or wild type α -synuclein results

in rounded, fragmented mitochondria *in vivo* and *in vitro* and this effect appears to be independent of Drp1 (Nakamura et al., 2011) while an age dependent decrease of Mfn1 and 2 has been observed in mice overexpressing a mutant form of α -synuclein suggesting a pathological role for the protein (Xie and Chung, 2012). Overexpression of LRRK2 has been shown to elicit mitochondrial fragmentation and increased mitochondrial localization of Drp1. This effect was attenuated with expression of specific PD-associated mutant forms of LRRK2 (R1441C and G2019S) in both SH-SY5Y cells and primary cortical neurons providing a direct link between PD and altered mitochondrial dynamics (Wang et al., 2012). Thus, it appears that a subtle effect on mitochondrial function, such as a decrease in CI activity, has a cumulative effect resulting a severe parkinsonian phenotype. Disruptions in the core systems of the ETC and mitochondrial dynamics overwhelm the capacity of mitochondria to compensate for additional insults and DA neurons may be more susceptible to these insults because of their pacemaker properties requiring a larger amount of ATP than most other cells in the brain.

1.4.3 Mitochondrial Dysfunction and Other Neurodegenerative Disorders

There are a wide variety of other neurodegenerative disorders associated with mitochondrial dysfunction, many of which are thought to be associated with mutations in genes with roles in mitochondrial housekeeping including Amyotrophic lateral sclerosis (ALS), Charcot-Marie Tooth Disease (CMT), Huntington's Disease and Spinocerebellar Ataxias (SCA). ALS is an adult onset neurodegenerative disorder that involves selective death of both peripheral and central motor neurons. As with other neurodegenerative disorders, 10-15% of cases are familial and about 25% of these are caused by mutations in the antioxidant enzyme superoxide dismutase (SOD1). It has been shown that both anterograde (De Vos et al., 2007) and retrograde (Shi et al., 2010) mitochondrial transport was reduced in motor axons of mice expressing mutant forms of SOD1. These revelations were supplemented by findings by Bosco et al. (2010) who took

misfolded wild-type SOD1 from a subset of patients with sporadic ALS and perfused it into isolated squid axoplasm where it inhibited fast axonal transport, providing a link between familial and sporadic disease pathology. HD is an autosomal dominant neurodegenerative disorder caused by an expanded CAG repeat in the first exon of the huntingtin (Htt) gene and is characterized by uncontrolled movement, dementia and premature death. Transfection of mutant Htt into rat primary cortical neurons blocks mitochondrial movement (Chang et al., 2006). Huntingtin associated protein (HAP1) is a binding partner of Htt and is known to associate with membranous organelles including mitochondria and has been shown to interact with kinesin and dynein to regulate transport of mitochondria on microtubules suggesting this may be the reason for disrupted mitochondrial trafficking (Bossy-Wetzel et al., 2008). HD patients also display altered mitochondrial dynamics (Oliveira, 2010) while expression of Drp1 is increased but expression of fusion related proteins is reduced in the striatum and frontal cortex (Shirendeb et al., 2011).

In conclusion, there is a large body of evidence linking mitochondrial dysfunction with almost all neurodegenerative disorders but there is no definitive answer as to whether impaired bioenergetics is the primary cause of adult onset disorders. The most likely scenario is a gradual change in mitochondrial function over the course of a person's life results in a reduction of the absolute production of ATP and at some point, this will result in bioenergetic failure. For example, if there was a slight dysfunction in the mitochondrial quality control process, the number of functionally defective mitochondria would slowly increase over time to a much greater extent in those neurons generating more mutated mtDNAs (in the hippocampus or SN) causing functional perturbations and neuronal cell death. Further investigation into mitochondrial dynamics is required and from these investigations one can envision a therapeutic target that could accelerate the removal of damaged mitochondria, increase fusion rates or increase $\Delta\Psi_m$ which would increase the pool of healthy mitochondria and potentially reduce neuronal death.

1.5 Glycosylation: The O-GlcNAc modification

Glycosylation is the biological process in which a carbohydrate is covalently attached to a polypeptide, lipid or other organic molecules through an enzyme catalysed reaction. One specific type of glycosylation is the O-linked β -*N*-acetyl glucosamine (O-GlcNAc) modification. Despite being a relatively recent discovery, protein modification by the addition or removal of O-GlcNAc has emerged as a major cellular signaling mechanism. It is an uncharged acetylated hexosamine sugar that is added to and removed from nucleocytoplasmic and mitochondrial target proteins by the intracellular enzymes O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) respectively (Bond and Hanover, 2015). There are many parallels between O-GlcNAc cycling and another common form of post-translational modification; phosphorylation. O-GlcNAc is also added to serine and threonine residues, the modification rapidly cycles on and off modified proteins at a rate faster than protein turnover, and like kinases and phosphatases, OGT and OGA are phosphorylated (Butkinaree et al., 2010). OGT uses the nucleotide sugar uridine diphosphate-O-GlcNAc (UDP-GlcNAc), which is synthesized in the hexosamine biosynthetic pathway (HBP), to catalyze the addition of O-GlcNAc to its target. UDP-GlcNAc availability is nutrient sensitive as glucose, acetyl-CoA, ATP, glutamine and uridine (all part of the HBP) are required for the synthesis of UDP-GlcNAc (Janetzko and Walker, 2014). Transcripts encoding O-GlcNAc cycling are most highly expressed in immune cells while high expression is also seen in the brain and the pancreas. As with other post translational modifications, O-GlcNAc cycling has been linked to roles in numerous human diseases including lupus, AD, autism and X-linked Parkinson dystonia (Bond and Hanover, 2013). Functions of O-GlcNAc cycling include the regulation of transcription and translation, the regulation of protein trafficking and turnover as well as underlying causes in diabetes, glucose toxicity and neurodegenerative disease.

1.5.1 The O-GlcNAc Cycling Enzymes: OGT and OGA

OGT is ubiquitously expressed within organisms and highly conserved across species. Many factors affect the regulation of OGT, including splice variants, PTMs and nutrient availability. The human OGT gene is located on the X chromosome near the *Xist* locus, a region which is associated with Parkinson's disease (Shafi et al., 2000), and three different isoforms may be generated through alternative splicing all of which contain an identical C-terminal catalytic domain, a linker region and N-terminal tetratricopeptide repeats (TPRs) (Love et al., 2003). The most abundant isoform is nucleocytoplasmic OGT (ncOGT) which has twelve TPRs, while an intermediate form known as mitochondrial OGT (mOGT) has nine TPRs and a mitochondrial targeting sequence at its N-terminus, and finally a short isoform (sOGT) with only two TPRs (Lazarus et al., 2006). However, recent research has suggested that only ncOGT is required for O-GlcNAcylation of mitochondrial proteins meaning mOGT is redundant and indeed not expressed in many species (Trapannone et al., 2016). OGT activity is highly dependent on the concentration of UDP-GlcNAc highlighting its role as a sentinel for nutrient availability in the cell. As GlcNAc is transferred to its target protein, UDP is released and acts as negative regulator of OGT activity showing fluctuating levels of UDP-GlcNAc are vital for regulating OGT activity (Hart, 2014). It is an inverting glycosyltransferase so it converts the α -linked GlcNAc of its donor to a β -linked PTM on its substrates (Drula et al., 2014, Coutinho et al., 2003). The process of O-GlcNAc addition appears to work via a bi-bi mechanism where UDP-GlcNAc binds the catalytic site first, followed by the polypeptide acceptor (Lazarus et al., 2012). In one instance, OGT has also been shown to act as proteolytic enzyme on host cell factor-1 (HCF-1), a transcriptional co-regulator of human cell cycle progression that undergoes cleavage as a maturation step. UDP-GlcNAc is required as a co-substrate for this reaction and to date is the only known cleavage reaction catalysed in the active site of a glycosyltransferase (Kötzler and Withers, 2016).

The human *oga* gene is found on chromosome 10q24.1, which is associated with increased risk of Alzheimer's disease (Twine et al., 2011) and gives rise to a 916

amino acid enzyme with two isoforms (Braidman et al., 1974). The nuclear isoform (ncOGA or OGA-S), has 662 amino acids is mainly found in lipid droplets and appears to have a primary role in proteasome regulation (Keembiyehetty et al., 2011). The full length isoform (OGA-L) has both nuclear and cytoplasmic localization and is ubiquitously expressed (Wells et al., 2002). OGA-L contains a catalytic glycoside hydrolase domain and a domain resembling histone acetyltransferases (HATs) but this domain lacks key residues thought to be required for an active acetyltransferase and most studies have not detected and HAT activity by OGA (Butkinaree et al., 2008, Schüttelkopf and Dorfmueller, 2013). Similarly, to OGT, the function and substrate specificity of OGA are controlled by interactions with a variety of cofactor proteins, often forming stable multiprotein complexes with each other.

1.5.2 Functions of O-GlcNAc

O-GlcNAc is broadly conserved across animals, plants and some fungi and has been shown to regulate a wide variety of cellular processes including transcription, cell metabolism, cell cycle progression and cell death (Hart et al., 2011). Proper O-GlcNAc cycling has been shown to be essential for survival in mammals as OGT knockout (KO) mice die at the one-cell stage and OGA KO mice die as neonates with developmental delays (Yang et al., 2012, Shafi et al., 2000). O-GlcNAc modifies all four core protein histones and competes with phosphorylation and ubiquitination which are associated with activation and repression of transcription (Lewis and Hanover, 2014). O-GlcNAc may also act as a link between glucose metabolism and cancer onset by modulating the stability of transcriptional activator β -catenin in a nutrient dependent manner (Stichelen et al., 2014). O-GlcNAc has also been shown to modify HIF-1 α and its transcriptional target GLUT1 which allows it to regulate glycolysis in cancer cells and ER stress, providing further evidence for altered O-GlcNAc cycling in cancer development (Ferrer et al., 2014). O-GlcNAc has also been shown to alter the activity of protein complexes as well as individual proteins. Researchers have discovered that O-GlcNAc modification of SNARE protein SNAP-29 correlates

with inhibition of autophagosome-lysosome fusion which opens up the possibility for a role for O-GlcNAcylation in synaptic transmission (Guo et al., 2014).

Despite recent evidence that the mitochondrial isoform of OGT appears to be redundant in many species, there is much evidence for an important role for O-GlcNAcylation in mitochondrial function. OGT binds to the histone deacetylase complex SIN3A at a site distinct from sites for binding histone deacetylase which allows a coordination of nuclear and mitochondrial gene expression (Kadamb et al., 2013). Elevated O-GlcNAc has been shown to modulate the recruitment and activity of Drp1 as increased O-GlcNAcylation at threonine 585 and 586 decreases phosphorylation at S637 augmenting the levels of the GTP-bound active form of Drp1 and inducing translocation from the cytoplasm to the mitochondria (Gawlowski et al., 2012). As mentioned above (section 1.3.1), increased O-GlcNAcylation of the mitochondrial adaptor protein Milton can diminish mitochondrial motility and so decrease rates of mitochondrial fusion in neurons (Pekkurnaz et al., 2014). Finally, studies have shown that many components of the OXPHOS system are O-GlcNAcylated (88 mitochondrial proteins in total) and this may have a profound effect on bioenergetic function in cells (Ma et al., 2015).

1.5.3 O-GlcNAc and Neurodegeneration

In recent years, O-GlcNAc cycling has become an intense area of focus for research into the pathology of neurodegenerative diseases because of the discovery that many pathogenic protein aggregates appear to have altered O-GlcNAcylation levels (Yuzwa et al., 2014). Defects in glucose metabolism occur in patients exhibiting Mild Cognitive Impairment (MCI), glucose hypometabolism is an early pathological change with AD brain and type 2 diabetes mellitus (T2DM) is a strong risk factor for the development of AD. As a result of the reduction in glucose metabolism, it has been shown that the levels O-GlcNAcylation in AD patient brains decrease to nearly half the levels in control brains. Kim et al. (2013) found that a specific inhibitor of OGA, NButGT, reduces A β production by

lowering γ -secretase activity *in vivo* and *in vitro* as well as attenuating the accumulation of A β , neuroinflammation and memory impairment in a mouse model of AD. In addition, Tau is extensively O-GlcNAcylated with an average stoichiometry proposed to be greater than four moles of GlcNAc per mole of tau protein (Arnold et al., 1996). It appears that the addition of O-GlcNAc to tau regulates the extent to which tau is phosphorylated, whereby an increase in O-GlcNAc results in a decrease in phosphorylation (Horsch et al., 1991). Yuzwa et al. (2012) showed that treatment of tau transgenic mice with thiamet G (an OGA inhibitor) increased O-GlcNAc, hindered formation of tau aggregates and decreased neuronal cell loss. The same authors also showed that treatment with thiamet G decreased the phosphorylation of Tau in PC12 cells at pathologically relevant sites including Thr231 and Ser396 indicating the reciprocal relationship between O-GlcNAc and phosphorylation (Yuzwa et al., 2008). These two residues are known to be key priming sites modulating the pathological hyperphosphorylation of tau in AD and the subsequent aggregation of NFTs highlighting the need for more investigation into the possibility of using OGA inhibitors as a therapeutic target in AD.

O-GlcNAcylation of various proteins has been implicated in a number of other neurodegenerative disorders. In ALS, the light and medium chains of neurofilament proteins (NF-L and NF-M), which make up part the axonal spheroids that accumulate in neurons, are both O-GlcNAcylated and phosphorylated (Honchar et al., 1982). Another protein heavily implicated in ALS is SOD1 has also been shown to be O-GlcNAc modified and mice expressing a mutant form of SOD1 have a global reduction of O-GlcNAc levels in their spinal cords implicating this post translational modification in the progression of ALS too (Chen et al., 2005, Shan et al., 2012). Finally, the modification has been identified in the protein most closely associated with PD, as α -synuclein has been shown to have a number of O-GlcNAcylation sites (Cole and Hart, 2001). Recent work has demonstrated that a modified form of α -synuclein with increased O-GlcNAc does not accelerate the aggregation of the protein whereas the analogous unmodified peptide does facilitate the aggregation (Marotta et al., 2012).

In conclusion, recent research has highlighted a potentially vital role for the post-translational modification of O-GlcNAc cycling in neurodegenerative disorders. It seems O-GlcNAc may play a generally protective role within the brain because of its high expression in this organ. This function may diminish in the aging brain, potentially due to a decrease in glucose metabolism, and may contribute to the progression of neurodegeneration through the increased aggregation of toxic protein oligomers or interference in correct mitochondrial function leading to cell death. The importance of O-GlcNAc in regulating protein stability is a fascinating subject and much more research is required to determine the true function of the modification, particularly in proteins associated with these debilitating disorders.

1.6 Glycosylation: Cell surface glycocalyx of neurons

In the previous section, it was explained how research on the O-GlcNAc post translational modification has accelerated in recent decades but the same can be said for the general field of glycobiology as until the 1980's carbohydrates were primarily considered as a source of energy or structural materials. This was in part due to the difficulty in determining sequences, structural complexity and because their biosynthesis could not be directly predicted from a DNA template. Advances in biochemistry have meant the field now encompasses the chemistry of carbohydrates, the enzymology of glycan formation and degradation, the recognition of glycans by specific proteins and glycan roles in complex biological systems, the latter two of which are of specific interest to this project. What follows is a brief overview of the post-translational modification which form glycoconjugates (glycoproteins and glycolipids) and the functions of these modifications.

The defining event in the biogenesis of a peptide-linked oligosaccharide is the formation of the sugar-amino acid bond; this will determine the nature of the carbohydrate units formed that will ultimately influence the proteins biological activity (Spiro, 2002). The most basic unit of a carbohydrate is a monosaccharide which is a carbohydrate which cannot be hydrolysed into a simpler form. Several hundred distinct monosaccharides are known to occur in nature but only a small number are known to occur in mammalian glycans. They are pentoses, hexoses, hexosamines, deoxyhexoses, uronic acids and sialic acids. Focus will be on the synthesis and functions of hexosamines such as GlcNAc, GalNAc and ManNAc as modified forms of these simple sugars will be used to examine the cell surface glycocalyx of primary cortical neurons (Wiederschain, 2009). There are at least 37 different glycan-protein linkages, which together involve 8 different amino acids and 13 different monosaccharides with the 5 groups named above (Lommel and Strahl, 2009). Protein glycosylation is classified into 5 major types based on how sugars are connected to amino acid side chains; *N*-glycosylation, *O*-glycosylation, glypiation, *C*-glycosylation and phosphoglycosylation. *N*-

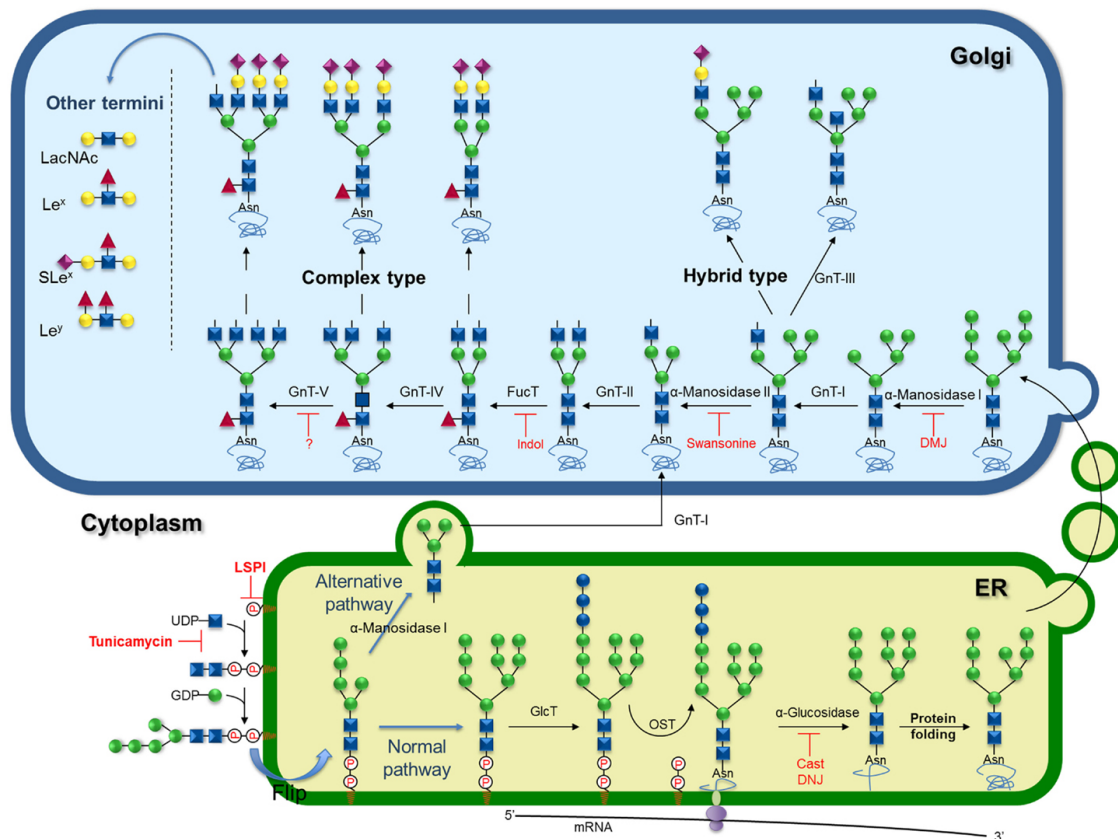


Figure 1.5 - Representative image of biosynthesis and processing of N-linked oligosaccharides. Image showing the biosynthesis of N-linked oligosaccharides starting in the ER before transport into the *cis*-Golgi and processing here. Also shown are common inhibitors of glycosylations and key targets for inhibition. Glycans are targeted to specific proteins and lipids in the Golgi. Taken from Vasconcelos-dos-Santos and Oliveira (2015).

glycosylation involves the addition of a sugar to nitrogen (N) atoms, usually to the nitrogens of asparagine side chains. All *N*-glycans share a common pentasaccharide core structure: GlcNAc₂Man₃ and this core structure is usually heavily modified and elongated after its introduction to the protein. In *O*-glycosylation, glycans are attached to side chain oxygens (O) of hydroxyl amino acid residues, often serine and threonine. *O*-glycosylation does not use a single monosaccharide and is initiated by quite a few carbohydrates including *O*-GlcNAc, *O*-*N*-acetyl-galactosamine (*O*-GalNAc), *O*-mannose (*O*-Man) and *O*-xylose (*O*-Xyl). *O*-GlcNAc glycosylation is discussed in more detail in section 1.5 and typically remains as a single monosaccharide residue but *O*-GalNAc, *O*-Man and *O*-Xyl can be elongated and branched to form complex structures (Peter-Katalinić, 2005). The other 3 forms of protein or lipid glycosylation are not relevant to this project and so will not be discussed here.

It is important to note that glycan chain structures are not encoded directly in the genome and are secondary gene products but around 700 proteins are needed to generate the full diversity of mammalian glycans which are estimated to be greater than 7000 structures (Cummings, 2009). Around 200 of these proteins are glycosyltransferases; enzymes which extend acceptor glycan structures using nucleotide or lipid linked sugars as activated donor substrates. Glycosyltransferases that possess overlapping glycan acceptor preferences but different donor specificities compete with each other and so can influence the abundance of glycan structures in the total glycome of the cell. Therefore, even with complete knowledge of the expression of all enzymes involved in building glycan structures, it is not possible to predict the exact structure generated in a given cell type. Subtle changes in the environment can produce dramatic changes in glycans produced by any given cell making total glycosylation a dynamic and powerful way to generate biological diversity and complexity (Wiederschain, 2009). This complex process can be initiated in the cytosol or nucleus in the case of *O*-GlcNAcylation (Bond and Hanover, 2015), but more commonly begins in the endoplasmic reticulum and moves from there through the various compartments of the Golgi apparatus before being distributed to various destinations from the

trans-Golgi network. Firstly, the sugar nucleotides are synthesized (as shown in Fig 1.6) within the cytosol and specific antiport proteins in the membranes of the ER and Golgi catalyse the import of the sugar nucleotides into the lumen of the organelle and the export of free nucleotides such as UMP, CMP and GMP which are generated within these organelles (Elsner et al., 2003). Numerous studies indicate that glycosyltransferases segregate into distinct compartments with the secretory pathway whereby enzymes acting in the beginning of the pathway are localized to the *cis* and *medial* compartments of the Golgi while those acting later co-localize in the *trans*-Golgi cisternae and the *trans*-Golgi network (Wiederschain, 2009). Three models have been proposed to account for the localization of glycosyltransferase to specific Golgi compartments. The first, the oligomerization/kin-recognition model, hypothesizes that enzymes involved in glycosylation form homo- or hetero-oligomers through interactions between their transmembrane and luminal sequences before arriving at the correct Golgi compartment. This oligomerization would serve to enhance the efficiency of sequential glycosylation reactions and has been shown to stabilize localization to specific compartments but only for a small number of enzymes (Nilsson et al., 1993). The second model, the lipid-partitioning model, hypothesizes that each glycosyltransferase sorts itself into the proper Golgi location based on the length of its transmembrane segment which is vital for retaining the enzyme once it reaches the correct compartment. This model was proposed based on an observation that the lipid bilayers in the Golgi stack increase in thickness in the direction of *cis* to *trans*. However, there does not appear to be a consistent relationship across glycosyltransferases between length of transmembrane segment and retention in a specific Golgi compartment (Patterson et al., 2008). In the third model, a new Golgi cisterna containing cargo molecules forms at the *cis* face and matures as Golgi glycosylation enzymes that define each sub-compartment are transported into the new cisterna from the old cisterna to modify the cisternal cargo proteins. Retrograde transport is at the core of maintaining a steady state localization in this model (Spang, 2013). Taken together, it is likely these 3 models overlap somewhat in explaining the continuous building of glycan

[illegible]

structures on glycoconjugates as they pass through the ER and Golgi but localization of glycosyltransferases within the Golgi is an important part of this process.

1.6.1 Functions of Glycosylation

As is the case with most other post-translational modifications, functions of glycosylation can range from the subtle to absolutely necessary for cellular and organismal growth, development and survival. The biological role of glycosylation can be subdivided into two broad categories; (1) structural and modulatory and (2) recognition of glycans by other molecules such as glycan binding proteins (GBPs). GBPs themselves can also be further subdivided into proteins that recognise glycans from the same organism and those which recognise glycans from different organisms which give them a role in immunity.

The structural role of glycosylation is most often mediated by attachment to matrix molecules, such as proteoglycans, which maintain tissue structure, porosity and integrity. In addition to this, the location of a majority of glycoconjugates on the cell surface provides a general shield, protecting the underlying polypeptide layer from recognition by proteases or antibodies. More strikingly, there are many examples of the role of glycosylation in modulating protein interaction and function at a variety of stages of differentiation. For example, neural cell adhesion molecule (NCAM) has chains of polysialic acids attached to it. In the embryonic state or other states of neural plasticity these anionic chains tend to be very long and interfere with homophilic binding thus inhibiting the normal function of NCAM which is to mediate homophilic binding between neuronal cells (Albach et al., 2004). Another function of glycans falling within this bracket is to act as a protective storage depot for biologically important molecules. Heparin-binding growth factors are found attached to glycosaminoglycan (GAG) chains of the extracellular matrix adjacent to cells that need to be stimulated, for example in the basement membrane underlying epithelial and endothelial cells, which prevents diffusion away from the site, protects against nonspecific proteolysis and allows

them to be released under specific conditions (Sasisekharan et al., 2002). Glycans can also act as specific ligands for cell-cell and cell-matrix interactions. For example, the selectin family of adhesion molecules specifically recognize glycan structures on their ligands and mediate interactions between blood cells and vascular cells in a wide variety of normal and pathological situations (Wiederschain, 2009). The biological significance of a number of these interactions has yet to be identified but it has become apparent that GBPs and glycans present on cell surfaces can interact specifically with molecules in the matrix or even with glycans on the same cell surface. The extrinsic recognition patterns of glycans is important from a biological perspective as certain sugars can act as specific binding sites for a variety of viruses, bacteria and parasites. This type of interaction typically has excellent recognition specificity of the glycan sequence. One such example exists in the hemagglutinins of many viruses recognize the type of host sialic acid, its modifications and its linkage to the underlying sugar chain. The evolutionary advantage of this specificity is not immediately obvious but if a pathogen was to first bind to a specific glycan ligand attached to a soluble mucin in the mucosal cell membrane which could then be washed away, it would remove any potential damage. There is also evidence for glycan binding mediates a symbiotic relationship between bacteria in the gut and the host organism (Bäckhed et al., 2005).

1.6.2 Glycosylation of neurons

Defects in different glycosylation pathways result in over 100 rare human genetic disorders and a large proportion of these impact the central nervous system with patients presenting with developmental delays, hypotonia, seizures, neuropathy and metabolic abnormalities in multiple organ systems. The reasons for the neuronal system being so heavily impacted is not fully understood but gated ion channels are heavily glycosylated, contributing 5-50% of their molecular weight and sialylation is especially significant (Ednie and Bennett, 2011). Defects in both *N*-glycosylation and *O*-glycosylation result in a neurological phenotype and both will be examined below.

The most common glycosylation disorder arises from mutations in phosphomannomutase 2 (*PMM2*) which converts mannose-6-phosphate to mannose-1-phosphate, which goes on to generate GDP-Man and dolichol-P-Man, the primary mannosylation donors. The mutation results in reduced levels of these molecules which leaves many proteins with only partially occupied N-glycosylation sites, decreasing the stability of these proteins (Freeze, 2013). Despite the large number of patients, little is known about how the mutations affects the nervous system as specific hypoglycosylated proteins have not been identified and phenotype ranges from moderate to death in patients with the same *PMM2* mutations (F119L, R141H). However, a common feature of the disease in children is cerebellar atrophy and research has shown that cerebellar granule cells are more sensitive than cortical neurons to inhibition of N-glycosylation by tunicamycin or *PMM2* knockdown and that this is due to increased ER stress in the cerebellar neurons (Sun et al., 2013). Myasthenic syndrome is a disorder that impairs signal transmission at the neuromuscular synapse and can be caused by mutations in genes involved in UDP GlcNAc synthesis but also in genes encoding lipid linked oligosaccharide (LLO)-mannosyltransferase such as *ALG2* (Cossins et al., 2013). It is not entirely clear how mutations in mannosyltransferases contribute to this phenotype but it is likely that addition of glycans stabilise acetylcholine receptors in the post synaptic membrane or play a role in neurotransmitter vesicle docking/fusion at the pre-synaptic site owing to the efficacy in using acetylcholinesterase inhibitors in treating this disorder. The above disorders arise as a result of malfunctions in N-linked glycosylation but there are also neurological conditions arising from disruption to O-linked glycosylation such as O-mannose glycosylation. The O- α Man glycans contain GlcNAc, Gal, GalNAc, Xyl, glucuronic acid and Sia in a complex pathway (Stalnaker et al., 2011). The major carrier of these glycans is alpha dystroglycan (α DG) which plays a crucial physiological role in neuromuscular junctions and linking skeletal muscle cell cytoskeleton to the extracellular matrix molecule, laminin. α DG O-Man forms an integral part of the dystrophin-glycoprotein complex that binds to laminin. Defects in this pathway result in a group of

disorders known as α -dystroglycanopathies and range from severe musculo-oculo-encephalopathies [including Walker Warburg syndrome (WWS)] to milder limb-girdle muscular dystrophy. WWS causing genes are all associated with O-Man structure formation and some are also linked to *N*-glycosylation (Jae et al., 2013).

To date, few critical target proteins which have a role in the neurological phenotype of glycosylation disorders have been identified. What has become apparent is that patients have hundreds of misglycosylated products, which affect a myriad of processes, including cell signalling, cell-cell interaction and cell migration. These phenotypes identify an important role for glycosylation in correct neuronal development and function. Our study will investigate any changes in neuronal cell surface glycocalyx throughout the differentiation process and identify the major pathways involved in the synthesis of cell surface glycoconjugates in neurons.

1.7 Substrate Transport into the Mitochondrion

Fatty acid oxidation is an important provider of substrates for the TCA cycle but transport of fatty acids into the mitochondria is an important step in this process. Pyruvate, fatty acids and amino acids are the main substrates for energy metabolism in the majority of tissues. Brain cells in particular, appear to be somewhat reliant on pyruvate produced directly as a result of glycolysis due to the apparent low activity of enzymes involved in β -oxidation in neurons (Panov et al., 2014). All of these substrates are transported to and broken down in the mitochondrion where the majority of ATP is produced through OXPHOS (Saraste, 1999).

Pyruvate lies at the heart of carbohydrate, fat and amino acid metabolism and is produced in the cytoplasm before being transported to the mitochondria, where it plays a role in lipid synthesis and gluconeogenesis (Daniel et al., 2012). Pyruvate transport into the mitochondrion is facilitated by the mitochondrial pyruvate carrier (MPC) but the full complement of genes that encode this carrier have yet to be identified (Hildyard and Halestrap, 2003). Andrew (2012) identified 3 genes conserved from yeast to man and confirmed that Mpc1, Mpc2 and Mpc3 were involved in this process by measuring UK5099-inhibitable pyruvate uptake into isolated mitochondria from different yeast strains. Expression of the mouse orthologs mMpc1 and mMpc2 were also shown to rescue yeast deletion mutants. Evidence from blue native gel electrophoresis suggests that Mpc1 and Mpc2 exist as part of 150kDa complex which confirms there are proteins which exist in this complex that have yet to be identified (Andrew, 2012). It is difficult to accurately measure mitochondrial pyruvate transport because it has is rapidly metabolized once inside the mitochondrion and because it occurs extremely quickly and on top of a significant rate of free diffusion across the mitochondrial membrane. However, the recent studies mentioned above have confirmed the importance of Mpc1/2 in this complex.

Transport of fatty acids into the mitochondria for β -oxidation is mediated by a carnitine carrier system, which is also thought to be the rate-limiting step in fatty acid oxidation. This transport system is made up of three proteins, carnitine palmitoyltransferase I (CPTI), carnitine:acyl-carnitine translocase (CACT) and carnitine palmitoyltransferase II (CPTII) (Kerner and Hoppel, 2000). The first step involves the transesterification of acyl-CoA to acylcarnitines in the MOM. CACT then catalyzes an exchange reaction whereby long chain acylcarnitines are translocated into the mitochondrial matrix. Finally, CPT-II, an enzyme located at the MIM, reconverts the acylcarnitine to the respective acyl-CoA so β -oxidation can continue (Currie et al., 2013).

CPT1 is the main rate-limiting step in β -oxidation and represents a key regulatory site controlling flux through this pathway by virtue of its inhibition by malonyl-CoA (McGarry and Mannaerts, 1977). The N terminal of CPT1 is essential for the inhibitory activity of malonyl-CoA and this system is part of a fuel sensing gauge, turning on and off fatty acid oxidation depending on the tissue's energy demands (Janos and Charles, 2000). There are 3 isoforms of CPT1; CPT1a is expressed most highly in the liver but also in pancreas, kidney and blood tissues, CPT1b is expressed only in brown adipose tissue, muscle and heart and CPT1c is expressed only in neurons (Lee and Wolfgang, 2012) (Sierra et al., 2008). There are some conflicting reports regarding the β -oxidation activity of CPT1c but Sierra et al. (2008) have shown that palmitoyl-CoA is the substrate in PC12 cells indicating it appears to play some role in neuronal oxidative metabolism. Further research is required to confirm its primary function and whether it is involved in β -oxidation in the brain and this study aims to examine the effects of inhibition of this pathway on mitochondrial dynamics.

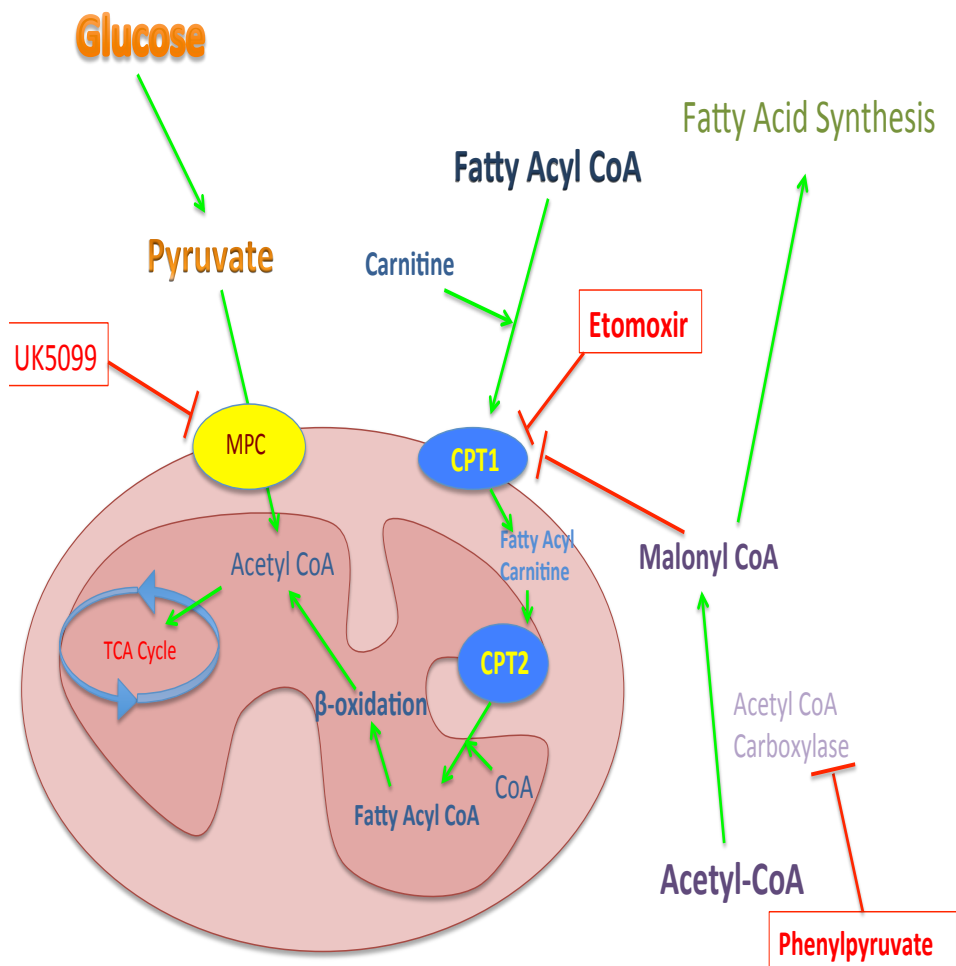


Figure 1.7 – Mechanisms of Substrate Transport into the Mitochondrion. Figure showing how the transport of pyruvate into mitochondria is mediated by the mitochondrial pyruvate carrier (MPC), a complex of 150kDa with an unknown of protein subunits. Fatty Acyl CoA enters the mitochondrion through the carnitine system, the rate limiting step of which is transport across the outer membrane by carnitine palmitoyl transferase 1 (CPT1). Both substrates are metabolized and enter the TCA cycle.

1.7.1 Acyl CoA Dehydrogenase 9

The acyl-CoA dehydrogenases (ACADs) are enzymes that catalyze the α,β -dehydrogenation of acyl-CoA esters in fatty acid and amino acid catabolism. β -oxidation of fatty acids is a spiral reaction of four enzymatic steps that takes place in mitochondria and peroxisomes. The first of these steps is the rate-limiting step and is catalyzed by enzymes from two protein families, the ACADs and the acyl-CoA oxidases (ACOXs). In mitochondria, ACADs transfer electrons from their corresponding CoA ester substrates to the electron transferring protein (ETF), which in turn transfers the electrons to ETF dehydrogenase. This is coupled with the ETC via CoQ to CIII for the production of ATP. In peroxisomes, the ACOXs transfer electrons directly to molecular oxygen producing H_2O_2 . All of these enzymes utilize FAD as a cofactor and are structurally similar (Swigonová et al., 2009). There are 11 members of the ACAD family contained in the human genome, each with unique substrate specificity and tissue expression profiles. Four of these are involved in β -oxidation of fatty acids with substrate specificity based on the length of the carbon chain; SCAD (short-chain acyl CoA dehydrogenase), MCAD (medium chain acyl CoA dehydrogenase), LCAD (long-chain acyl CoA dehydrogenase) and VLCAD (very long-chain acyl-CoA dehydrogenase) (Zhang et al., 2002). ACAD9 bears much functional similarity to VLCAD and retains the ability to carry out β -oxidation but this is not thought to be its primary role as will be discussed below. Four other members of the family are involved in catabolism of amino acids while the functions of the two most recently discovered members of the family, ACAD10 and ACAD11, have yet to be elucidated but have high expression levels in the brain (He et al., 2011). Mitochondrial β -oxidation of long chain fatty acyl-CoAs is a primary metabolic pathway for maintenance of energy homeostasis and body temperature but many patients with defects in this respect present with neurological disease suggesting that β -oxidation in the CNS is involved in functions alternative to energy metabolism, many of which have yet to be elucidated (Vockley and Whiteman, 2002).

ACAD9 was first discovered by Zhang et al. (2002) via large scale random sequencing of a human dendritic cell (DC) cDNA library, where they described a gene with 47% amino acid identity and 65% similarity with VLCAD. ACAD9 consists of 18 exons and 17 introns mapped to chromosome 3q26, with an open reading frame of 1866 bp encoding a protein of 621 amino acids. They showed high expression levels in the heart, skeletal muscle, brain, kidney and liver and demonstrated the recombinant ACAD9 protein has the highest dehydrogenase activity on palmitoyl-coenzyme A (C16:0) and stearoyl-coenzyme A (C18:0). At this point there was no evidence to suggest that ACAD9 had any other role other than in β -oxidation but recent studies have suggested that this may be an evolutionary conserved mechanism and the true function of the protein lies elsewhere. Nouws et al. (2010) set out to find proteins involved in the regulation of CI biogenesis by screening for proteins associated with CI subunits NDUFAF1 and Ecsit and found ACAD9 as a candidate for CI assembly. Despite the fact ACAD9 mutations had been implicated in fatty acid oxidation disorders (He et al., 2007), they noticed that ACAD9 had a number of specific characteristics associated with OXPHOS genes; its promoter has a sequence homologous to the nuclear respiratory factor 1 (NRF1), it includes cAMP-responsive element transcription factor binding (CREB) sites and during mitochondrial import it is processed by a two-step cleavage, the second of which is processed by mitochondrial intermediate peptidase (MIP), which mostly processes proteins involved in the OXPHOS system (Chew et al., 2000). These observations suggested that ACAD9 could have a role in CI assembly and this hypothesis has gained strength with a number of recent results. Catalytically inactive ACAD9 gives partial-to-complete rescue of CI biogenesis in ACAD9-deficient fibroblast cell lines and was incorporated in high molecular weight assembly intermediates (Nouws et al., 2014). Knockdown of ACAD9 resulted in a reduction in expression of other CI proteins including NDUFAF1 and Ecsit, suggesting the improper or non-assembly of CI results in the degradation of these proteins (Scheffler, 2010). CI deficits have also been shown in HEK293 cells in which ACAD9 has been knocked out and these effects can be rescued by wild type ACAD9 (Schiff et al., 2015). However, this paper also demonstrated that knockout of ACAD9 affects

long-chain fatty acid oxidation suggesting that there is a role for ACAD9 in fatty acid oxidation in tissues where it is highly expressed. They also showed that there is a significant inverse correlation between residual ACAD9 activity and the phenotypic severity of ACAD9 deficient patients. These findings were replicated by Nouws et al. (2014) showing that the enzymatic role of ACAD9 is not redundant *in vivo* and that it may provide a link between the two major metabolic pathways; fatty acid oxidation and oxidative phosphorylation. Thus, the current hypothesis is that in cells that do not express ACAD9 highly, it has a sole function in CI assembly and its contribution to fatty acid oxidation is negligible whereas in tissues where it is expressed highly it serves a dual function. This opens up many questions regarding the use of fatty acids as an energy source in the brain, which was not previously thought to occur.

ACAD9 dysfunction has been implicated in a number of disorders, highlighting its dual function. He et al. (2007) first described a number of patients with ACAD9 deficiency who presented with episodic liver dysfunction and chronic neurological dysfunction. All patients presented with marked deficits in the ACAD9 protein that could not be compensated for by the actions of VLCAD. Haack et al. (2010) identified five pathogenic ACAD9 alleles with a causative role in Complex I deficiency in a screen of 120 complex I-defective cases. CI deficiency is a frequent biochemical condition, accounting for about one third of mitochondrial respiratory chain disorders that compromises the transfer of electrons derived from carbohydrates and fatty acids to CoQ. Finally, Thomas et al. (2012) examined frontal cortex regulation of mitochondrial biogenesis and its effects on CI in PD patients and found widespread decreased expression of nDNA CI genes. Interestingly, they showed that ACAD9 gene expression in brains of PD patients was reduced to about 20% of the levels seen in the frontal cortex of control brains, while only moderate decreases were seen in other CI assembly factors NDUFAF1 and Ecsit. This highlights the importance of ACAD9 in CI assembly and suggests a potential causative role for ACAD9 downregulation in PD.

1.8 Aims of the Thesis

Mitochondrial dynamics has been demonstrated to be an important part of mitochondrial function in a variety of cell types. In particular, neuronal cells appear to be especially susceptible to alterations in mitochondrial dynamics due to their high energy demands and abnormal morphology. This is evident in the continuing discussion of the causes of a wide variety of neurodegenerative disorders where mitochondrial dysfunction is a frequently recurring theme. Many studies have implicated defects in ETC complexes in neurodegenerative diseases but little correlation has been drawn between these and mitochondrial fusion in neuronal cells or a neuronal model. In addition, newer areas of research such as O-GlcNAcylation of mitochondrial proteins have been implicated in the aforementioned conditions but little research has been carried out to examine these effects on mitochondrial dynamics and bioenergetic function. To address these issues, this study aimed to achieve the following:

- Quantify the effects of ETC inhibition on mitochondrial fusion in primary neurons and differentiated PC12 cells and to correlate these effects with any alterations in $\Delta\Psi_m$ and ROS production.
- To investigate the existence of a threshold effect of complex I inhibition on mitochondrial fusion and identify underlying causes for this.
- To assess the effects of pyruvate transport and fatty acid metabolism on mitochondrial dynamics and mitochondrial function in differentiated PC12 cells.
- To examine the effects of altered O-GlcNAc levels on mitochondrial dynamics and bioenergetics function using both pharmacological methods and gene knockdown.
- To examine the make-up of neuronal cell surface glycocalyx using confocal microscopy.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Materials

ADP, ATP, agarose, ammonium persulfate, ampicillin, antimycin A, ascorbic acid, bovine serum albumin, calcium chloride, cytochrome *c*, decylubiquinone, dipotassium hydrogen phosphate (monobasic), dimethyl malonate, D-glucose, dichloroacetate, DMSO, DNase, ethanol, EDTA, EGTA, etomoxir, FCCP, fetal bovine serum, ferricyanide, Ficoll, glutamate, HEPES, horse serum, magnesium chloride, magnesium sulfate, malate, methanol, NADH, NADP⁺, Nancy-520, nerve growth factor, penicillin-streptomycin solution, percoll, peroxidase from horseradish, phosphate buffered saline, phosphoenol pyruvate, poly-D-lysine, potassium cyanide, potassium EDTA, potassium phosphate, rotenone, sodium chloride, sodium pyruvate, succinate, sucrose, tetramethylethylenediamine, thiamet G, Trizma base, trypsin-EDTA, trypsin inhibitor, UK-5099, veratridine were all purchased from *Sigma Aldrich, Poole, UK*.

ProtoGel[®] was obtained from *National Diagnostics, USA*.

Coomassie (Bradford) Protein Assay Kit, dipotassium-EDTA, Lipofectamine[®] 2000, OptiMEM and Trypan blue solution were obtained from *Thermo Scientific, UK*.

EMD Millipore Immobilon[™] Western Chemiluminescent HRP substrate (ECL) was obtained from *Millipore, Ireland*.

Miniprep, Maxiprep, and RNA isolation kits were purchased from *Qiagen, Hilden, Germany*

Antibodies used were anti-Opa1 antibody from *BD, UK*; anti-Drp1 antibody from *Millipore, Ireland*; anti-Mfn1 and anti-Mfn2 antibodies from *Santa Cruz, USA*; anti-beta Actin, anti-O-linked N-Acetylglucosamine and anti-Ndufa9 antibodies from

Abcam, UK; anti-synaptophysin and anti-ACAD9 antibodies from *Proteintech, UK*. Peroxidase-conjugated AffiniPure Goat Anti-Rabbit and Anti-Mouse IgG secondary antibodies were obtained from *Jackson ImmunoResearch Laboratories, USA*.

ST045849 (TT04) was purchased from *TimTec, USA*.

Vectors pDsRe2d-mito and PA-GFP-mito were obtained from *Addgene, UK*.

RPMI 1640 + GlutaMAX[®], B27 and alamarBLUE[®] were purchased from *Biosciences, UK*.

2.2 Preparation of Solutions and Pipetting

Reagents were weighed on a Mettler College Model analytical balance for weights above 5g or on a Mettler K7t top-loading balance for weights below 5g. All aqueous solutions were prepared using deionized water from a Millipore Elix advantage 10 water purification system. All solutions were adjusted to the required pH using a Corning pH meter, Model 240, which was calibrated daily using standard buffer solution of pH 4.0, 7.0 and 10.0. Gilson pipettes were used to pipette volumes from 200nl to 5ml.

2.3 Buffers

STE buffer: 320 mM sucrose, 10 mM Trizma-Base and 1 mM EDTA, pH 7.4.

Percoll isolation buffer: 315 mM mannitol, 75 mM sucrose, 1 mM EGTA, 20 mM HEPES, pH 7.2.

Krebs buffer: 3 mM KCL, 140 mM NaCl, 25 mM Tris – HCl. 10 mM glucose, 2 mM MgCl₂, pH 7.4.

Complex I buffer: 5 mM KH₂PO₄, 10 mM MgCl₂, pH 7.4.

Complex II/III buffer: 100 mM KH₂PO₄, 0.3 mM K – EDTA, pH 7.4.

Complex IV buffer: 100 mM KH₂PO₄, pH 7.4.

TAE buffer: 40 mM Tris – acetate, 2 mM EDTA, pH 8.0.

Running Buffer: 25 mM Tris – Base, 190 mM glycine, 0.1% SDS, pH 8.3.

Anode buffer 1: 300 mM Tris – Base, 10% methanol, pH 10.4.

Anode buffer 2: 25 mM Tris – Base, 10% methanol, pH 10.4.

Cathode buffer: 25mM Tris – Base, 200 mM glycine, pH 9.4.

RIPA buffer: 150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8.0.

2.4 Centrifugation

For volumes of 1.5ml or less, an Eppendorf Benchtop Centrifuge 5415 was used. Some high-speed spins were required for the maxi prep and these were carried out in a Sorvall RC-5B centrifuge with a SS34 rotor. For the purpose of pelleting cells in volumes of 2-50ml an Eppendorf Centrifuge 5810R was used.

2.5 Electrophoresis

An AE-6450 Dual Mini Slab Kit and an Atto Crosspower 3500 were used for all SDS-PAGE electrophoresis.

2.6 Experimental Animals

All experimental animals were obtained from the Comparative Medicine Unit, Trinity College Dublin, Ireland. Neonatal pups, weighing approximately 20g each, were sacrificed at postnatal day 1 and killed by swift beheading using sharp, sterile, surgical scissors. Female Wistar rats weighing ~250g were used for preparation of rat brain mitochondria and were sacrificed by stunning followed by cervical dislocation.

2.7 Cell Culture

PC12(6-15) cell line was obtained as a gift from Dr. Rainer Pepperkok (EMBL, Heidelberg, Germany). Cells were maintained in RPMI 1640 and supplemented with 10% Fetal Bovine Serum, 1% Penicillin-Streptomycin and 1% L-Glutamine.

For differentiation, PC12(6-15) cells were maintained in RPMI 1640 and supplemented with 1% Fetal Bovine Serum and 100ng/ml Nerve Growth Factor (NGF) while all other supplements remained the same. Cells were maintained in this medium for 48 hours until complete differentiation was achieved and experiments were carried out. All cell culture work was carried out under sterile conditions in a NUAIRE laminar flow hood. All cells were maintained in at 37 °C, in 95% humidity and 5% CO₂. Cell growth and viability was monitored visually using a Nikon Eclipse TS100 light microscope with 10x and 20x dry objectives. Cell counts were performed using an Invitrogen Countess Automated Cell counter and cell viability was assessed by exclusion of trypan blue dye.

2.8 Transfection

PC12 (6-15) cells were transfected using Lipofectamine 2000 transfection reagent. Cells were plated at a density of 3×10^5 cells/well of a 6 well plate the day before transfection was due to take place. On the day of transfection, complete RPMI 1640 medium was replaced with antibiotic free medium (all other supplements the same). Lipofectamine (6µl) was added to a sterile eppendorf tube containing 250µl of OptiMEM, mixed and incubated at room temperature for 5 minutes. In another sterile eppendorf tube, 2-3µg of plasmid DNA was added to 250µl of OptiMEM. After 5 minutes, the OptiMEM/Lipofectamine mix was added to the OptiMEM/DNA mix and the complete transfection solution was incubated at room temperature for 20 minutes before being added directly to the cells. After 4 hours, the transfection/medium mixture was replaced with fresh complete RPMI 1640 and cells were allowed to recover overnight. The following day cells were plated on 35mm µ-dishes and differentiated for imaging 48 hours later.

2.9 Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 6. All data sets were tested for Gaussian distribution and equal variance within the GraphPad Prism software. All data are presented as mean $\pm$ SEM unless otherwise stated. A P-value of 0.05 or less was deemed to be statistically significant. Unpaired t-tests

were performed to analyse samples of no more than two different groups. One-way ANOVA followed by Dunnett's multiple comparison tests were used for samples with more than two groups. Two-way ANOVA followed by Dunnett's multiple comparison tests were used for samples containing more than two groups that were split into two variables.

2.10 Image Processing

All images were processed using a combination of FV10-ASW Olympus Fluoview Ver. 2 software (for confocal microscopy images), Amaris software (for confocal images taken using the newer Leica system), Leica SP8 software, ImageLab Ver. 5 software and Apple Preview.

2.11 Protein Extraction

Protein was extracted from PC12 cells and primary cortical neurons using RIPA buffer to lyse the membranes. Cells were washed in PBS 3 times, scraped and spun down. They were then resuspended in 100µl to 500µl of RIPA buffer containing protease inhibitors depending on the volume of cells in the pellet. Cells were agitated at 4°C for 30 mins before being spun down at 3000rpm at 4°C for a further 20 mins. The supernatant was removed to a fresh eppendorf tube and protein estimation was carried out as per the method of Bradford (1976) with modifications.

2.11.1 Bradford Assay

Bovine serum albumin served as a standard and was prepared from a stock of 10mg/ml as a series of dilutions ranging from 50-500µg/ml with a final volume of 1ml (in triplicate). 10µl of these and of diluted samples of unknown protein concentrations were added to 200µl of Commassie Protein Assay Reagent in a 96 well microtitre plate and samples were incubated in the dark for 15 minutes. After this incubation, the absorbance of each was read at 595nm in a SpectraMAX

PLUS Microplate Spectrophotometer, corrected for blank. An example of the standard curve commonly generated is given in Fig. 2.1.

2.12 Western Blotting

For SDS-PAGE analysis, samples were added directly to 5X Sample Buffer to give a final concentration of 1X sample buffer. Samples were then heated at 65°C for 15 minutes. 10% and 12% polyacrylamide gels were used for Western Blots depending on the size of the protein to be analyzed and were prepared using the recipe established in the Davey laboratory.

	Stack	Resolve	
	4%	10%	12%
Water (ml)	2.7	4.0	3.3
30% Acrylamide Mix (ml)	0.67	3.3	4.0
1.5M Tris (pH 8.8) (ml)		2.5	2.5
1.0M Tris (pH 6.8) (ml)	0.5		
10% SDS (ml)	0.04	0.1	0.1
10% Ammonium Persulfate (ml)	0.04	0.1	0.1
TEMED (ml)	0.004	0.004	0.004

Table 2.3.1 - Recipes for SDS-PAGE

Resolving gel acrylamide polymerization was initialized by the addition of TEMED, mixed and poured into an empty gel cassette. To ensure a level boundary between the stack and resolving gel was overlaid with H₂O saturated butanol. Once the gel had set, the H₂O saturated butanol was washed off using distilled water and filter paper was used to remove any excess. The stacking gel was then polymerized by the addition of TEMED, mixed and poured on top of the resolving gel. A comb was inserted before the gel had set to form the wells for loading samples. Once the gel had set, it was inserted into a vertical electrophoresis tank immersed in 1X Running Buffer. Depending on the antibody, 20 or 30 µg of

protein sample was loaded into each well and samples were flanked by Spectra Broad Range Multicolour Protein Ladder (BioRad), in order to determine the progress of protein migration. Gels were run at a constant current (20mA/gel) for 90 minutes using an Atto Crosspower 3500 PowerPac.

Proteins resolved by SDS-PAGE were transferred onto Immobilon-P PVDF membrane (Millipore) by semi-dry transfer technique. Briefly, the PVDF membrane was activated in methanol and then pre-soaked in Anode II buffer. A sandwich was created whereby One sheet 3MM filter paper was placed in Anode I buffer, 2 sheets of 3MM filter paper soaked in Anode II buffer were placed on top of this followed by the activated PVDF membrane, the SDS-PAGE gel and 3 pieces of 3MM filter paper which had been soaked in Cathode Buffer. Proteins were transferred at constant voltage (10V) for one hour. Following transfer, membranes were blocked in 10% non-fat dried milk (NFDM) solution in TBS-T overnight in a 50ml Falcon tube on a roller at 4°C to reduce non-specific antibody binding. After blocking, membrane was washed briefly with TBS-T and primary antibodies were diluted in 5% NFDM solution in TBS-T for 2 hours at room temperature. After primary antibody incubation, membranes were washed 3 times (15 minutes each) in TBS-T and secondary antibodies were diluted in 5% NFDM solution in TBS-T for 1 hour. Membranes were washed again before the antibodies were detected by the addition of Enhanced Chemi-Luminescence (ECL) for 3 minutes. Signal generated by the HRP substrate was then detected using a chemi-luminescent gel documentation system (BioRad). Images generated by the BioRad system were analysed using the ImageLab Software.

2.13 Confocal Microscopy

Cells (fixed and live) were imaged using an Olympus FV1000 Point scanning confocal microscope, FV10-ASW Olympus Fluoview Ver. 2 software and a 60X oil immersion objective and later a Leica TCS SP8 STED microscope with associated software and 60X oil objective. Sequential excitation at 405nm, 488nm and 546nm were provided by near-violet laser diode, argon and helium-neon gas

lasers respectively. The imaging chamber was heated to 37 °C, humidified and contained 5% CO₂ for live cell imaging.

2.13.1 Quantification of Mitochondrial Fusion

The method of quantification of mitochondrial fusion was based on that of Karbowski et al. (2004), with modifications, and relied on the principle that mitochondria share matrix contents upon fusion. By conferring high fluorescence to a small group of mitochondria within the cellular mitochondrial matrix (photoconverting mitochondria-expressed GFP to a state of ~20-fold increased fluorescence in a small region of interest, or ROI), mitochondrial fusion can be monitored by measuring the rate of decrease in fluorescence intensity in the ROI as matrix contents are shared by fusing organelles. All experiments were carried out using the Olympus FV1000 point scanning Confocal Microscope, FV10-ASW Olympus Fluoview Ver. 2 software and a 60X oil immersion objective with 2X zoom applied or the Leica TCS SP8 confocal microscope, Leica SP8 software and a 60X oil immersion objective set with 2X zoom applied. Cells cultured in 35mm glass bottomed μ -dishes were placed in the live cell-imaging chamber and covered with the CO₂ regulator/humidifier apparatus. Sequential excitation at 488nm and 542nm allowed for visualization of PA-GFP and DsRed protein expression in transfected cells respectively. Using the software's Multiple Time Lapse facility, five separate fields were pre-selected per group (control and treated) per dish, each containing at least one cell. Cells were imaged with z-stacking (6-7 slices of 0.8 – 1.4 μ m thickness) before and after photoactivation of discrete 6.25 μ m² regions of interest (ROIs) of the mitochondrial network, with post activation imaging intervals set at 1, 15, 30 and 45 minutes. Photoactivation was achieved by 5 second irradiation of ROIs using the 405nm laser set to 1% power output. The photoactivated protein exhibited ~90% increased fluorescence compared to its non-activated state. Regions exhibiting less than 75% increase in fluorescence post-activation were excluded from studies, as were cells that had clearly entered mitosis when differentiation had begun. Using the software's Image Analysis facility, the mean pixel intensities of the photoactivated ROIs were

calculated in both the red and green channels at all time points and expressed as a percentage of pixel intensity 1 minute post activation, deemed to be the point at which pixel intensity was highest. Pixel intensities were also measured in three 6.25 μm^2 ROIs in cell free areas and subtracted from the cellular ROIs as “background” fluorescence. Each treatment was repeated on at least three separate dishes of cells on three separate occasions (n=3); thus, each data point on graphs depicting fusion rates is representative of data from at least 15 cells.

2.13.2 Quantification of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured using the $\Delta\Psi\text{m}$ – dependent dye tetramethylrhodamine, methyl ester (TMRM). This cationic, red-orange fluorescence-emitting dye is sequestered by mitochondria in a $\Delta\Psi\text{m}$ dependent manner and at non-quenching concentrations (below 50 nM), indicates mitochondrial depolarisations upon loss of signal (and conversely, hyperpolarization upon increase in signal). Cells were loaded with the dye in normal culture medium containing 20 nM TMRM for 30 minutes before being washed with HEPES-Tyrode buffer 3 times and incubated in this buffer with 5 nM TMRM for the duration of imaging. Excitation with the 543nm laser set to 1.4% power output allowed for the visualization of red-emitting TMRM within mitochondria of cells, viewed through a 60X oil objective. The imaging settings were adjusted such that the background fluorescence was negligible and the signal from cells was below saturation level. Five randomly chosen fields were pre-selected using the softwares’ Multiple Time Lapse facility and imaged before and after incubation with or without compounds. Using the softwares’ Image Analysis facility, mean pixel intensities of each field were calculated and expressed as a percentage of initial, pre-incubation values.

2.13.3 Immunocytochemistry

Cells cultured on 13mm glass coverslips were fixed in 4% paraformaldehyde in PBS for 15 minutes, permeabilized in 0.2% Triton in PBS for 10 minutes, rinsed with PBS and blocked (5% BSA, 1% FBS, 0.1% Triton in PBS) for 1 hour. Cells

were incubated with primary antibody in the above-mentioned blocking solution at 4°C overnight and in secondary antibody (anti-rabbit/mouse 488 or 593, Abcam) in the same blocking solution. Coverslips were placed on a small dot of ProLong Gold Antifade Mountant containing the nuclear counterstain 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen) or hydromount on glass slides and sealed with clear nail varnish, then stored at 4°C, in darkness, until imaging.

2.14 Molecular Biology

The pDsRed2-mito and PA-GFP-mito plasmids used encode a fusion fluorescent protein and mitochondrial targeting sequence from subunit VIII of human cytochrome c oxidase. Both contain cytomegalovirus (CMV) promoters and confer neomycin resistance in eukaryotic cells and kanamycin resistance in *E. Coli*. Although both plasmids were already present in the laboratory, it was necessary to prepare large stocks of each to ensure enough was available for all planned experiments as a transient transfection protocol was used. These stocks were prepared as below. All microbiological procedures were carried out using aseptic technique.

2.14.1 Transformation of Competent Bacterial cells

Five Hundred nanograms of plasmid DNA was added to 20µl Top 10 Chemically Competent *E.Coli* in a sterile 1.5ml minifuge tube. The bacteria were heated to 42°C for 90 seconds and then placed on ice for 30 minutes. 80µl of Luria Broth was added to the transformed cells and the tube was sealed and incubated at 37 °C with gentle agitation for one hour. Following this incubation, the bacteria were spread on a 9cm petri dish containing 12ml sterile Lennox LB Agar at 35g/L, containing 50µg/ml kanamycin. The plate was incubated overnight at 37 °C to allow for growth of colonies of successfully transformed bacteria.

2.14.2 Growth of Single Colonies and Miniprep

To increase the volume of cells from an individual colony for the purposes of performing a Miniprep and making glycerol stocks, 5ml of 20g/L LB Broth (containing 50µg/ml kanamycin) was inoculated with a single colony picked from agar plates using a sterile 2-200µl yellow tip in a 15ml Falcon tube. A total of 3 individual colonies were selected for amplification and incubated overnight with constant swirling (200rpm) at 37 °C. The following day, 1ml of each culture was set aside and stored at 4°C for making glycerol stocks, pending a successful miniprep. The remaining 4ml of each was centrifuged at 13000rpm for one minute in batches of 1.5ml, to pellet the cells. The Miniprep was then carried out using a Qiaprep Spin Miniprep Kit as per manufacturer's instructions.

2.14.3 Restriction Digest and Agarose Gel Electrophoresis

The final plasmid isolates produced in the Miniprep were assessed by agarose gel electrophoresis. For the restriction digest, 1µg plasmid DNA was incubated with 2.5% (v/v) BamH1 restriction enzyme in a final volume of 50µl digest buffer for 1 hour at 37 °C. To this, 5µl of 6X sample loading buffer was added and 25µl was loaded per well of a 1% (w/v) agarose gel (1g agarose in TAE buffer; 40mM Tris-HCl, 200mM Sodium Acetate, 1mM EDTA, pH 8.2) containing 0.01% (v/v) Nancy. These samples were run alongside a 1kb wide range DNA ladder. The gel was run at 100V for 75 minutes and the DNA bands were visualized under UVP Bioimaging Systems Chemi II Darkroom and Labworks Image Acquisition and Analysis Software V 4.0.0.8.

2.14.4 Glycerol Stocks and Maxiprep

Glycerol stocks were prepared by adding 20% (v/v) sterile glycerol to the 1ml of culture derived from the single colonies chosen and previously set aside before the miniprep. Stocks were frozen at -80°C until required. For the maxiprep, LB agar plates containing 50µg/ml kanamycin were streaked with *E. Coli* from

glycerol stocks and incubated overnight at 37 °C. The following day, a single colony was picked and inoculated with 5ml of LB broth in a 15ml Falcon tube. The tube was incubated at 37 °C for 8 hours, with swirling (200rpm), and then added to 195ml of LB Broth, containing 50µg/ml kanamycin, in a 200ml Erdinger flask and incubated overnight at 37 °C, with swirling. The cells were then pelleted by centrifugation at 4000rpm for 20 minutes. The plasmid was extracted using Qiagen's Plasmid Maxi Kit, according to manufacturer's specifications. Plasmid purity was assessed by ensuring that the ratio of Abs_{260/280} was less than 1.80, indicating minimal lipopolysaccharide contamination. This was performed using a NanoDrop – ND1000 spectrophotometer.

2.15 Generation of Stable Knockdown Lines

Small interfering RNA (siRNA) processed from short hairpin RNA (shRNA) constructs purchased from Sigma were used to mediate ACAD9, O-GlcNAc transferase and O-GlcNAcase RNA interference (RNAi) in PC12 cells. The constructs were provided in bacterial glycerol stocks and lentiviral transduction particles for immediate transduction into cells.

2.15.1 Puromycin Kill Curve

As the plasmid confers puromycin resistance in eukaryotic cells, a kill curve was constructed to ascertain the optimum concentration to use to select positively transfected cells. Cells were seeded in a 96 well plate at a density of 1.6×10^4 /well. The following day 200-2000ng/ml of puromycin was added to the cells and viability was examined every day thereafter. After 4 days it became obvious what the optimum concentration for completely killing all cells was and those concentrations which had no effect on cell viability. The appropriate puromycin concentration for stable cell line selection was deemed to be 800ng/ml.

2.15.2 Transduction of Cells

It was decided to use the lentiviral construct to achieve a high transduction efficiency with the siRNA. Cells were plated at a density of 7.5×10^4 /well in a 24 well plate, 24 hours prior to transduction to achieve a 70% confluency for transduction. The lentiviral stocks were thawed on ice and added to the cells at a suitable Multiplicity of Infection (MOI) based on the number of transducing units per millilitre as shown by a p24 antigen ELISA titre. The following day, the viral particle-containing medium was replaced with fresh complete RPMI 1640 to allow for recovery. Selection was started the following day with 800ng/ml puromycin and the cultures were grown up for 10-12 days before a sample was lysed and assayed for interference of the target gene.

2.16 Spectrophotometry

Different spectrophotometers were used for different assays. A Shimadzi UV-VIS spectrophotometer UV-2600 with different attachments was used to assay activities of the different ETC complexes. A SpectraMax M3 96-well plate reader was used to estimate protein concentration and H₂DCFDA assays.

2.17 Respiratory Chain Complex Activity Assays

2.17.1 Complex I Activity Assay

The complex I activity assay was based on a protocol described by Darley-USmar (1987). The assay measures the oxidation of NADH to NAD⁺ with concomitant decrease in absorbance at 340 nm at 37 °C. The reaction mixture contained assay buffer, 0.2 mM NADH, 2.5 mg BSA, 1 mM KCN and 0.05 mg/ml non-synaptic mitochondria (NSM) or cell lysate. The assay was started and the activity was measured for 2-3 min to make sure there was a stable baseline. Decylubiquinone (50 µM) was then added to each well to start the reaction in the spectrophotometer and the reaction rate was followed for 6-9 min. Rotenone (10 µM) was later added to obtain the rotenone sensitive rates and the reaction was

further followed for 5 min. the rotenone sensitive rates were subtracted from the decylubiquinone rates and the Beer-lambert law ($A = \varepsilon \times c \times l$) was used to obtain the specific activity of the enzyme. The specific activity was expressed as nmol/min/mg of protein.

2.17.2 Complex II/III Activity Assay

Complex II/III activity was measured using a method based on that of King (1967). The reaction follows the reduction of cytochrome *c* using succinate as the e^- donor, measured at $\lambda = 550$ nm at 37 °C. The reaction mixture contained assay buffer, 20 mM succinate, 1 mM KCN and 0.05 mg/ml NSM or cell lysate made up to a final volume of 1 ml in each cuvette. The reaction was started by the addition of 100 μ M cytochrome *c* and the resulting increase in absorbance was measured for 6-9 min. Antimycin A (1 μ M) was added to inhibit the reaction and the antimycin A sensitive rates were followed for 5 min. The antimycin A sensitive rates were subtracted from the initial rate and the specific activity of the enzyme was calculated using the Beer-Lambert law ($A = \varepsilon \times c \times l$). The specific activity of the protein was expressed as nmol/min/mg of protein.

2.17.3 Complex IV Activity Assay

The protocol used to measure complex IV activity was based on that of Wharton and Tzagoloff (1967). The assay follows the oxidation of reduced cytochrome *c* by complex IV (cytochrome *c* oxidase) and is measured as a decrease in absorbance at $\lambda = 550$ nm at 37 °C. The reaction mixture contained assay buffer and 50 μ M reduced cytochrome *c* with a final volume of 1 ml in the cuvette. The reaction was started by the addition of 0.05 mg/ml NSM or cell lysate to the cuvette and was followed for 10 min. the activity is non-linear and is therefore expressed as a first order-rate constant (k/min/mg).

2.18 ROS Measurement

The dye 2',7'-dichlorodihydrofluorescein di-acetate (H₂DCFDA) was used to measure ROS production in PC12 cells and primary cortical neurons. This dye gets oxidised into the fluorescent compound DCF in the presence of ROS in cells. PC12 (6-15) cells or cortical neurons were seeded in black 96 well plates coated with poly-D-lysine at a density of 30,000 cells/well and differentiated for 48 hours (8 days for neurons). Cells were washed once with warm PBS, before Krebs buffer containing 5 μ M H₂DCFDA was added to each well. Fluorescence was read over 1 hour at excitation λ =485 nm and emission λ =530 nm.

2.19 Cell Viability

To analyse cell viability, alamarBlue® was used, which contains the active ingredient resazurin, which gets converted to the fluorescent compound resorufin by living cells. PC12 (6-15) cells were seeded in 96-well plates coated with poly-D-lysine at a density of 30,000 cells/well and differentiated for 48 hours. AlamarBlue® was added to each well (10%) and incubated for 4 hours before fluorescence was measured at excitation λ =570 nm and emission λ =600 nm.

2.20 Respiration Measurements

A Seahorse extracellular flux analyser XF24 was used for measurement of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR). These measurements were used as an indicator of glycolysis and oxidative phosphorylation rates respectively. PC12 (6-15) cells or primary cortical neurons were seeded at densities of 100,000 cells/well or 200,000 cells/well and cultured for 2 or 8 days in a seahorse 24 well cell culture plate. 18 hours prior to the beginning of the experiment, a seahorse cartridge was hydrated using seahorse calibration buffer in a -CO₂ incubator at 37 °C. On the day of the experiment, seahorse media was supplemented with 11 mM glucose (and NGF or B27) and warmed to 37 °C. Cells were washed twice with warm seahorse media and then 600 μ l of seahorse media was added to each well, including blank wells for

background measurement. The plate was placed in a $-CO_2$ incubator for 45 min prior to initiation of the experiment. During this incubation, 70 μ l of inhibitors in seahorse media were added to designated ports of the seahorse cartridge plate at final concentrations as follows; oligomycin (2 μ g/ml), FCCP (1 μ M), rotenone (100 nM) plus antimycin A (2 μ M) and 2-DG (30 mM). The seahorse cartridge was then calibrated in the analyser and the cell plate was placed in the seahorse machine.

Chapter 3

Bioenergetic Control of Mitochondrial
Dynamics in PC12 Cells and Primary
Cortical Neurons

3.1 Introduction

Given the large size and distinct morphology of neuronal cells and their high bioenergetic demand, the roles of mitochondria within various cellular compartments can vary greatly, making mitochondrial dynamics particularly important in this cell type. As a result, subtle alterations in the dynamics machinery over time can have deleterious effects on mitochondrial and cellular function in the aging brain. This chapter deals with the effect of electron transport chain inhibition on mitochondrial dynamics in a neuronal model and neurons, with particular focus on complex I.

3.1.1 Mitochondrial Dynamics in Neurons

Perturbations in mitochondrial dynamics has been heavily implicated in the progression of neurodegenerative disorders (see section 1.4 for further details) but the importance of mitochondrial dynamics in neuronal cell survival, synaptic transmission and neural development has also been well documented (Flippo and Strack, 2017). A growth cone is an extension of a developing neurite seeking its synaptic target in which there is a high concentration of mitochondria in order to satisfy the high energy demands of these motile structures (Morris and Hollenbeck, 1993). Mitochondrial dynamics can influence the direction in which the growth cone develops by altering the balance between fission and fusion. Inhibition of Drp1 or Mfn2 overexpression, thus promoting mitochondrial elongation, was shown to enable the crossing of growth cones in rat retinal ganglion cells into stripes of inhibitory growth factors at the expense of crossing into stripes of permissive factors (Steketee and Moysidis, 2012). There is also a role for dynamics at the post synaptic density, where Li et al. (2004) demonstrated that activity dependent transport of mitochondria to dendrites is required for maintenance of dendritic spines in cultured hippocampal neurons. This effect was further highlighted in studies which showed that inducing mitochondrial fragmentation, by overexpression of Drp1, enhances synapse formation whereas inhibition of Drp1 has the opposite effect in cultured hippocampal neurons (Dickey

and Strack, 2011). Away from neuronal development, mitochondrial dynamics also play an active role in maintenance of synaptic transmission, which hints at why slight perturbations in the system may lead to onset of neurodegeneration in later life. In lamprey, frequently firing dorsal column synapses have approximately twice the number of mitochondria as phasic reticulospinal synapses which fire far less frequently (Brodin et al., 1999). Mitochondrial fission is necessary for dendritic localization of mitochondrial at the post synaptic density although they are not localized to the dendritic spine. This has a direct implication on memory formation as mitochondrial Ca^{2+} uptake at the post synaptic density has been shown to be necessary for long term potentiation (LTP) while ATP production at these sites is also a vital part of the process (Stanton and Schanne, 1986). Mitochondrial dynamics likely influence almost every aspect of neuronal function and as neurons cannot be easily replaced, they are especially sensitive to alterations in the dynamics system. As a result, mutations in the components of this system invariably result in neurological symptoms highlighting the importance of maintaining correct mitochondrial integrity and function in the brain.

3.1.2 Complex I in the Brain

Complex I is the largest of the complexes encompassing the ETC and consists of 46 subunit proteins, 7 of which are encoded by the mitochondrial genome (Smeitink et al., 2001). It exerts a high level of control over OXPHOS activity as it contributes to about 40% of the proton motive force for ATP synthesis and exhibits a low inhibition threshold, thus exerting a high level of control over synaptosomal bioenergetics (Galkin et al., 2006, Telford et al., 2009). CI activity is thought to vary between brain regions which may explain why certain areas of the brain, such as the hippocampus or the substantia nigra, are more susceptible to neurodegeneration. One study, by Pollard et al. (2016), showed that CI activity was higher in the cortex of brains isolated from young adult mice when compared to the activity from the cerebellum and brainstem. This study also demonstrated a decrease in CI activity in aged mice and in the brains of a mouse model of neurodegeneration which will be discussed further below.

Complex I assembly is complicated because of its large size, the number of subunits (45) and the fact that it is under the regulation of two genomes as 7 of the subunits are encoded by mtDNA. CI assembly is not sequential meaning each of the subunits is imported into the mitochondrion and intermediates are assembled independently and then joined, and sub-assemblies of the nuclear DNA encoded subunits and the mtDNA encoded subunits can be formed independently (Friedrich and Weiss, 1997, Lazarou et al., 2007). Given the large size of neurons and the relatively high rate of protein turnover, tight regulation of this process is also essential for correct mitochondrial function. CI is also most commonly assembled into supercomplexes in the neuronal mitochondria whereas greater amounts of free CI are found in astrocytes. This note is important as assembly into supercomplexes is associated with an increase in oxidative phosphorylation and also a decrease in ROS production (Lopez-Fabuel et al., 2016). Thus, incorrect or decreased CI assembly into supercomplexes could have severe consequences in terms of neuronal cell survival and oxidative damage in aged neurons. Taken together, these examples show the importance of correct complex I assembly and function in specific brain regions in maintaining neuronal cell health and identify a potential role for CI in maintenance of mitochondrial dynamics.

3.1.3 Complex I and Neurodegeneration

Initial studies that showed potential complex I involvement in neurodegeneration focused on PD arose after accidental infusions of MPTP and other inhibitors of complex I induce dopaminergic neurodegeneration in flies, humans and rodents (Langston et al., 1983, Chaturvedi and Beal, 2008). Further evidence has arisen in isolated mitochondria from the substantia nigra, skeletal muscles and platelets of PD patients which show reduced CI activity levels and in mice and rats which develop a robust PD phenotype after systemic rotenone treatment. There is also evidence of mitochondrial damage due to oxidative stress resulting in complex I mis-assembly in mitochondria isolated from the frontal cortex of PD patients,

further implicating CI in PD progression (Keeney et al., 2006). Finally, it has been well documented that CI inhibition leads to chronic production of ROS which can cause severe oxidative damage to susceptible mitochondria. Several studies have reported that neurons of the substantia nigra show evidence of oxidative damage and mutations in genes associated with antioxidant properties such as DJ-1 have been implicated as high-risk factors for onset of PD (Dauer and Przedborski, 2003, Bonifati et al., 2003).

Complex I dysfunction has also been linked to several other neurodegenerative disorders and the general aging process where it has been argued that a general decline of mitochondrial function is the primary driver of aging. Indirectly, complex I inhibition and reduced respiration has been linked to hyper-phosphorylation of tau, increasing its aggregation and contributing to Alzheimer's disease pathophysiology (Melov et al., 2007). Some evidence has also been found of reduced complex I activity in mitochondria isolated from platelets of patients with HD, although there is little in the literature detailing this effect in brain mitochondria of HD patients (Parker et al., 1990). Links between decreased CI activity and ALS have also been identified in mutant cell lines where activity was decreased by up to 20% but most studies on this disorder have found more significant defects in the activity of cytochrome c oxidase (complex IV) in brain tissue (Swerdlow et al., 1998). Other areas within the field of neuroscience have also begun to identify defects in OXPHOS and in complex I in a variety of other neuronal disorders, including schizophrenia (Prabakaran et al., 2004), which further highlights the importance of fully functioning neuronal metabolism in order to maintain brain health. Regardless of the site of the bioenergetics defect, all of these conditions display a phenotype in which there appears to be reduced oxygen consumption and an altered mitochondrial morphology, emphasizing the close relationship between bioenergetics and mitochondrial dynamics which has yet to be fully characterised. For the purposes of this study, much of the focus will be centred around complex I dysfunction and subsequent onset of parkinsonism.

3.1.4 α -synuclein and Mitochondrial Dynamics

As detailed in section 1.4, the main pathological hallmark of PD is an accumulation of α -synuclein in Lewy bodies in the substantia nigra and the cortex in later stages of the disease. α -synuclein is a presynaptic protein whose physiological function is not fully understood but appears to play a role in synaptic plasticity (Kaplan et al., 2003). It is a predominantly cytosolic protein but a fraction appears to be present in the mitochondria, the proportion of which increases when α -synuclein is overexpressed or when the cytoplasm is an acidic environment (Nakamura et al., 2008). The protein has been shown to associate with CI in the inner mitochondrial membrane and reduces CI activity when transfected into human dopaminergic neurons, which could lead to dysfunction of the mitochondrial fission/fusion machinery (Devi et al., 2008). Indeed, in mice expressing a mutant form of α -synuclein (A53T), there is an age dependent alteration in mitochondrial morphology and expression of the mitodynamins, while cellular models show decreased levels of mitochondrial motility (Xie and Chung, 2012). It has also been shown that overexpression of α -synuclein increased the translocation of Drp1 to the mitochondrial membrane, increasing its fission activity. This effect was mediated through a mechanism involving extracellular signal-regulated kinase (ERK) (Gui et al., 2012). These results show the impact α -synuclein has on mitochondrial function and morphology yet it has never been demonstrated if overexpression of the protein directly impacts mitochondrial fusion. This study will examine the effect of both wild type and mutant (A53T) α -synuclein overexpression on mitochondrial fusion in differentiated PC12 cells.

3.1.5 Aims of the Chapter

- To quantify the effects of ETC inhibition on mitochondrial fusion in differentiated PC12 cells.
- To find the biochemical causative factors of any inhibition of mitochondrial fusion and mitochondrial membrane potential, ROS production or post translational modifications of the fusion machinery.
- To quantify the relationship between complex I inhibition and fusion/fission thresholds in primary cortical neurons.
- To analyse Ndi1 restoration of mitochondrial dynamics in neurons where Ci activity has been inhibited.
- To quantify the effects of α -synuclein overexpression on mitochondrial dynamics in differentiated PC12 cells.

3.2 Methods

3.2.1 Primary Cortical Neuron Culture

Primary Cortical Neurons were isolated from the brains of one day old neonatal Wistar rats. Rats were decapitated using surgical scissors and the cortices carefully extracted using fine-tipped curved forceps. The tissue was cross chopped and incubated for 25 minutes in 0.3mg/ml trypsin/PBS, at 37 °C. The trypsinisation process was halted by the addition of increasingly concentrated solutions of trypsin inhibitor; PBS containing 0.03mg/ml soybean trypsin inhibitor, 0.06ml/ml, DNase and 3mM MgSO₄, followed by PBS containing 0.1mg/ml soybean trypsin inhibitor, 0.2mg/ml DNase and 10mM MgSO₄. The cortical suspension was re-suspended and passed through a 40µm nylon mesh strainer and the filtrates were centrifuged at 2000 x g for 3 minutes at room temperature. The resultant pellets were re-suspended in 1.5ml Neurobasal medium-A (NBMA, Invitrogen), supplemented with 10% horse serum (HS), 2mM L-Glutamine, penicillin-streptomycin solution (100 units/ml penicillin G, 0.1mg/ml streptomycin sulfate) and 2% B27 and counted, using trypan blue as a diluent. Each brain yielded approximately 3-5 x 10⁶ cells. Immediately after isolation, cells were plated in aforementioned medium, on 13mm glass borosilicate coverslips in 24 well plates or in 35mm glass bottomed µ-dishes (Ibidi, Germany) at a cell density equivalent to the total extract from one cortex per 24 well plate/per three 35mm µ-dishes. Each coverslip/dish was coated in 40µg/ml poly-d-lysine (MW 70-150kDa) for 2 hours prior to seeding of cells. After three hours, medium was replaced with NBMA supplemented with 1% HS (all other supplements identical). After 24h, cells were exposed to 20µM cytosine beta D-arabinofuranoside (Ara-C) in 1% HS NBMA to discourage the proliferation of glial cells. After a further 24 hours, Ara-C was removed and cells maintained in Ara-C-free NBMA (0% HS), with 50% medium changes every 3-4 days. Cells were used for experiments at DIV 8-11.

3.2.2 Transfection of Primary cortical neurons

Neurons were transfected directly after isolation from cortices using Amaxa's Rat Cortical Neuron Nucleofection System. Cells were transfected with 3µg of each plasmid (PA-GFP and DsRed) at 4×10^6 per transfection and plated on poly-d-lysine coated 35mm glass bottom µ-dishes at a density of 2×10^6 cells per dish. Three hours post nucleofection, medium (10% HS NBMA) was refreshed to remove cell debris. After 24h, cells were exposed to 20µM cytosine Ara-C in 10% HS NBMA to discourage the proliferation of glial cells. After a further 24h, Ara-C was removed and cells maintained in Ara-C-free 1% NBMA (0% HS), with 50% medium changes every 3-4 days. Cells were used for experiments at 8-11 DIV.

3.2.3 Preparation of Non-Synaptic Mitochondria

The protocol for preparation of non-synaptic mitochondria (NSM) was based on the method previously described by Lai and Clark (1989). Two rats were sacrificed, decapitated and the brains were removed and placed in a beaker containing ice cold STE buffer. The brains were finely chopped using scissors and let settle at the bottom of the beaker before the STE buffer was carefully decanted off and replaced with fresh STE. This process was repeated until most of the blood was removed. The chopped brains were homogenised using a handheld glass Dounce homogeniser by carefully applying passes of a loose-fitting pestle followed by a few passes of a tight-fitting pestle, until completely homogenised. The homogenate was divided in two 50 ml tubes, filled up with STE and centrifuged for 3 mins at 3000 rpm in a Sorvall centrifuge using an SS34 rotor, pre-cooled to 4°C. The resulting supernatant was transferred to two 50 ml tubes and balanced before centrifugation at 10,000 rpm for 10 min. the supernatants were carefully discarded and the crude mitochondrial pellets were pooled together and placed back into the homogeniser where 2-3 passes of the tight pestle were applied. The pellet was re-suspended in STE buffer up to a total volume of 9 ml and carefully placed on a discontinuous Ficoll gradient, consisting

of a 17 ml bottom layer of 10% (w/v) Ficoll and a 9 ml top layer of 7.5% (w/v) Ficoll, in a clear 35 ml ultracentrifuge tube. The homogenate was centrifuged in a pre-cooled (4°C) 6 x 35 ml swing-out rotor in a Thermo Scientific Sorvall WX Ultra Series Centrifuge at 28,000 rpm for 45 min with the acceleration and deceleration settings set to 1. The result from the centrifugation can be seen in Fig 3.1. The top layer of myelin was discarded with a Pasteur pipette before collecting the synaptosome fraction at the interface of the 7.5% and the 10% Ficoll layers, as well as collecting the NSM which made up a pellet at the bottom of the tube. They were suspended in separate 50 ml centrifugation tubes and centrifuged at 10,000 rpm for 10 min to remove any remaining Ficoll. The pellets were collected and the protein concentration was determined using a Bradford assay followed by either experiments or immediate freezing of the samples at -80°C for future use.

3.2.4 Generation of PC12 cells Stably Overexpressing α -synuclein

Work carried out in collaboration with Dr. Natalie Adlesic. Pegfp-C1 vectors expressing wild type or A53T mutant human α -synuclein were purchased from Addgene, USA, and the α -synuclein inserts were subcloned into pcDNAV5 His B vectors. This was done by adding NotI and HindIII sites to either side of the inserts by incorporating them at the end of each primer (Eurofins genomics) used to amplify the DNA through PCR. The DNA was purified and ligated into the pcDNA V5 His B vectors, which were also digested with NotI and HindIII and the resulting clones were verified by sequencing, and restriction digest with NotI and HindIII.

PC12 cells were transfected with either the pcDNA- α -syn-WT-V5 His B, pcDNA- α -syn-A53T-V5 His B vector or an empty pcDNA V5 His B vector as a control using lipofectmaine 2000. These vectors encoded an insert for blasticidin resistance, allowing for selection of stable transfected cells. The cells were seeded (1000 cells/well) in a 96 well plate and selected after blasticidin treatment (20 μ g/ml) and screened for α -synuclein overexpression by western blotting.

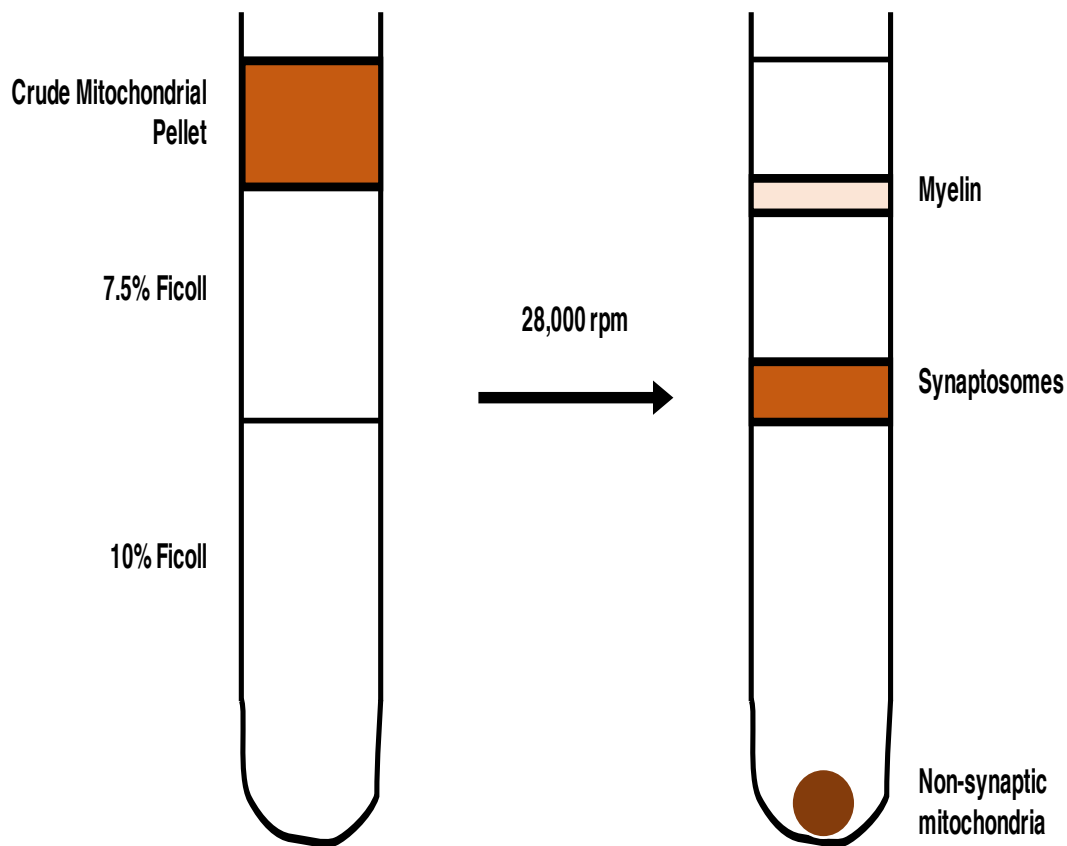


Figure 3.1. Ficoll Gradient for separation of crude mitochondrial pellet fractions. The Ficoll gradients before and after centrifugation at 28000 rpm for 45 min at 4°C. The sample consists of the crude mitochondrial pellet on top of 7.5% Ficoll, which is layered on top of 10% Ficoll in an ultracentrifuge tube. After centrifugation, Ficoll and myelin are found at the top of the tube. The synaptosomes are found in the interface of the 7.5% and 10% Ficoll layers. The nonsynaptic mitochondria are found as a pellet at the bottom of the tube.

3.3 Results

3.3.1 Mitochondrial Fusion in Differentiated PC12 cells and Primary Cortical Neurons

Mitochondrial fusion in differentiated PC12 cells and primary cortical neurons was assessed using live cell confocal microscopy. Cells were transiently co-transfected with two-expression plasmids encoding mitochondrial targeted fluorescent proteins. The first, DsRed-mito, confers red fluorescence to mitochondria allowing for visualization of the entire mitochondrial network and its morphology. The second, photo-activatable green fluorescent protein (PA-GFP-mito), undergoes photo-conversion upon irradiation with 405nm light and exhibits significantly increased fluorescence with excitation at 488nm (Patterson and Lippincott-Schwartz, 2002). This property allows for the photoactivation of discrete regions of interest (ROIs) in the mitochondrial network, the pixel intensity (PI) of which decreased as mitochondria actively fused and shared (diluted) the photo-activated protein. Thus, a decrease in mean PI in the ROI indicated active mitochondrial fusion. Conversely, the perturbation of mitochondrial fusion resulted in a maintenance or lesser decrease of mean PI over the course of an experiment.

Mitochondrial fusion in the cell somata of differentiated PC12 cells and primary cortical neurons were assessed under resting conditions and under conditions where mitochondrial function was disrupted through the use of inhibitors and uncouplers of the electron transport chain. At rest, differentiated PC12 cells exhibited active mitochondrial fusion, in general agreement with the published literature in the subject area (Lee et al., 2004) (Berman et al., 2009) (Cagalinec et al., 2013). For quantification of the fusion rates, the mean pixel intensities (PIs) of the photoactivated ROIs were calculated in both the green and the red channels before and after photoactivation (1,15, 30 and 45 minutes after photoactivation) and expressed as a percentage of PI values one minute post activation, deemed to be the point at which the PA-GFP-mito within the $6.25\mu\text{m}^2$

region of interest (ROI) was emitting at its highest possible level. Fifteen minutes after photoactivation, pixel intensities of the ROIs were reduced to an average of 65.532% of the value after one minute in PC12 cells and 72.19% in primary neurons. Thirty minutes after photoactivation, the pixel intensity (PI) was reduced to averages of 45.8% and 53.1% respectively. In certain cell types, such as cancer cell lines, the rates of mitochondrial fusion are higher and the experiment is stopped at this point but as the PI was still relatively high after 30 minutes, it was decided to monitor fusion for 45 minutes in total. As a result, average PI values dropped to 36.8% of the initial value 45 minutes after photoactivation in differentiated PC12 cells and 40.1% in primary cortical neurons, which can be observed in Fig 3.3. The PI of the DS-Red protein showed statistically insignificant fluctuations during this time period, indicating that the PI decrease observed in relation to the PA-GFP was not due to a large movement of mitochondria away from the ROI.

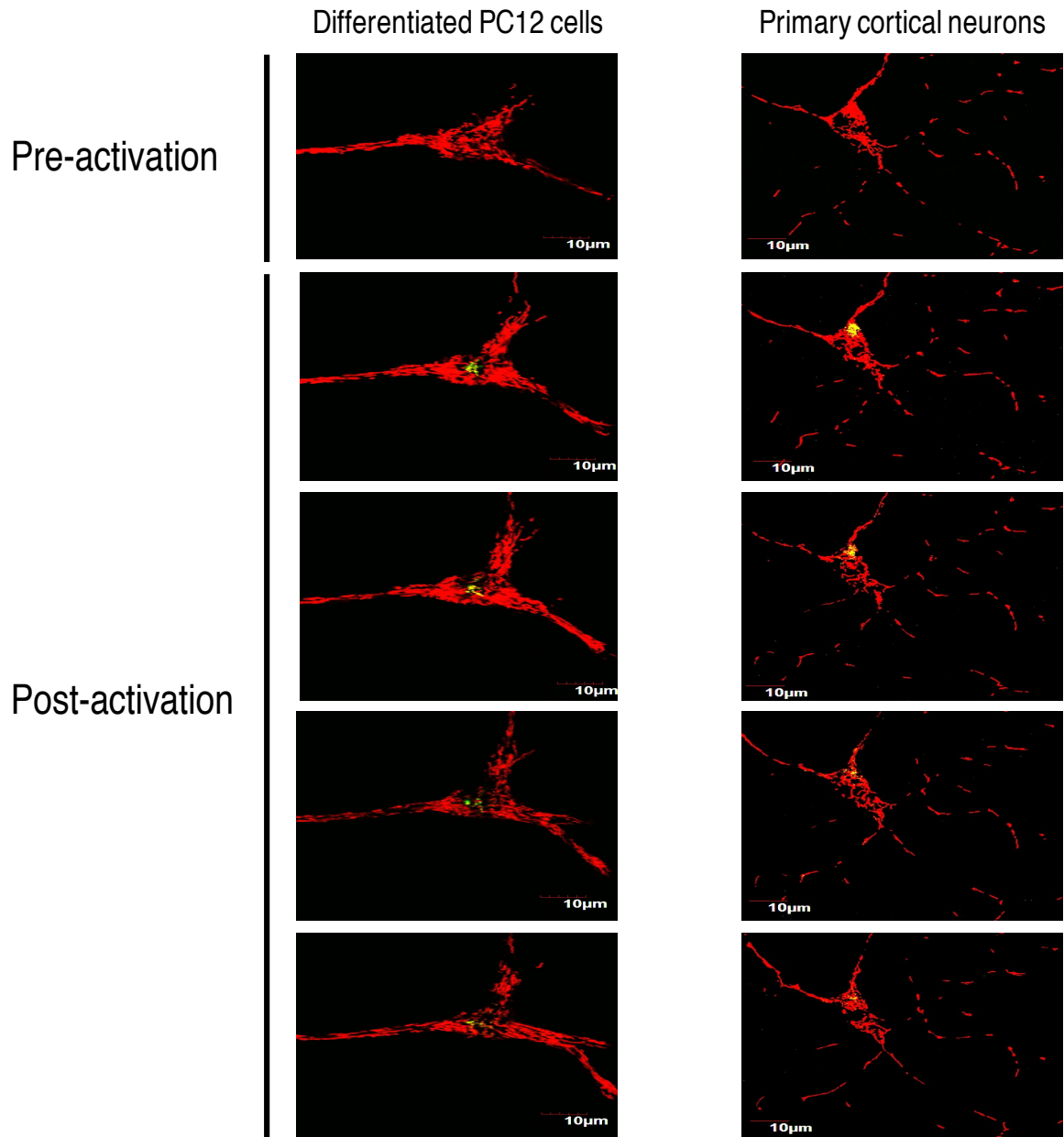


Figure 3.2: Representative image of mitochondrial fusion in the soma of differentiated PC12 cells and primary cortical neurons at resting conditions. Differentiated PC12 cells and primary cortical neurons were transfected with DS-Red and PA-GFP as described in sections 2.8 and 3.2.2 and analysed by confocal microscopy using an Olympus FV1000 and later a Leica Sp8 microscope. Photo-activated mitochondria of the cell soma show gradual sharing of fluorescent contents over the duration of a 45 min experiment.

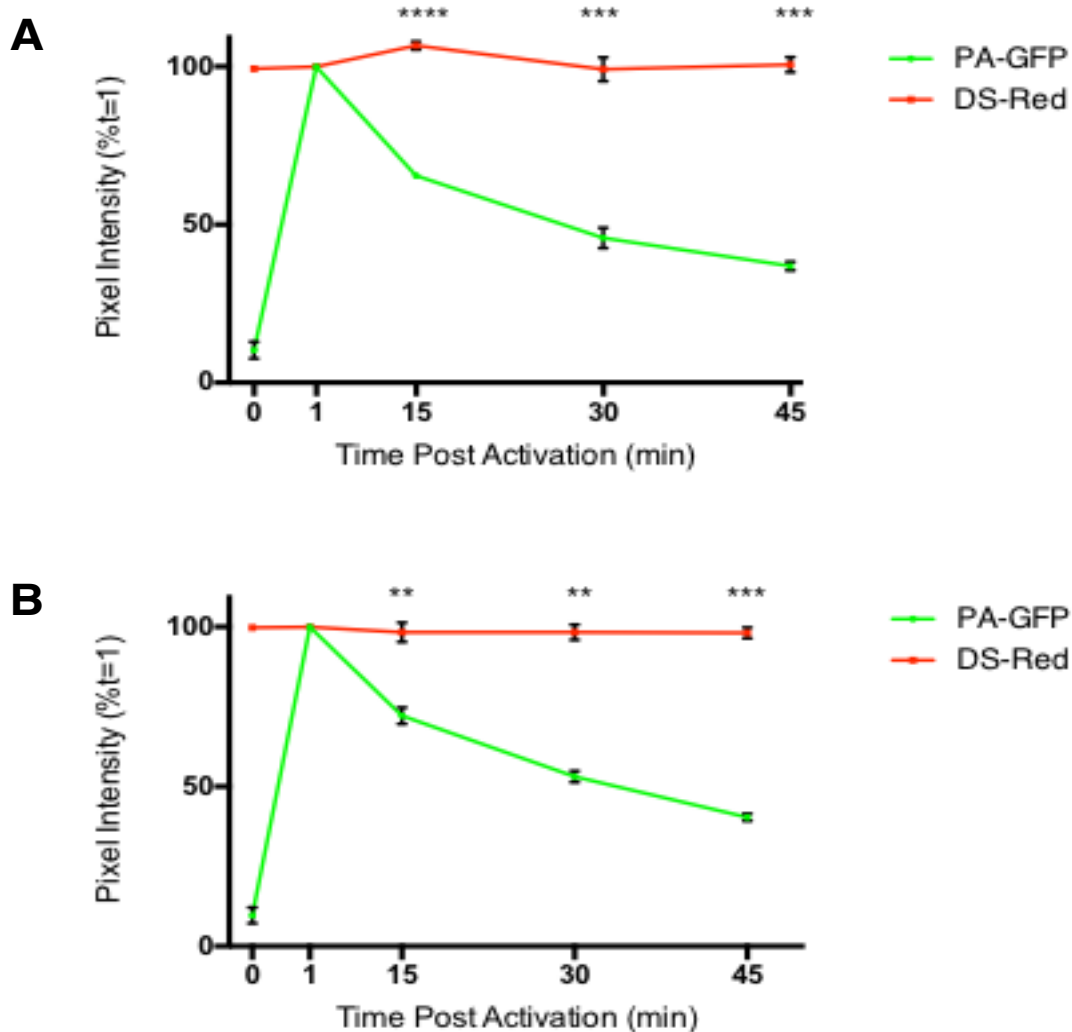


Figure 3.3: Graphical display of mean pixel intensity changes over time in photo activated ROIs of (A) differentiated PC12 cells and (B) primary cortical neurons.

Mean pixel intensities of $6.25\mu\text{m}^2$ photo-activated regions of interest (ROIs) were calculated, initially using Olympus FV10-ASM Fluoview Ver.2 software and later using Leica SP8 LAS X software, and expressed as a percentage of pixel intensity at 1 minute post activation ($t=1$) which was the point at which pixel intensity should be highest. In the PA-GFP channel, there is a low level of fluorescence prior to activation with a mean pixel intensity of $>10\%$ of that of its 1 minute post activation value. The value consistently reduces until it reaches $\sim 40\%$ of its $t=1$ level 45 min after photo-activation in both cell types. Throughout the experiment, the DS-Red channel showed statistically insignificant fluctuations in pixel intensity at the time points shown indicating the decrease in pixel intensity from the PA-GFP channel were not due to movement of mitochondria out of the ROI or photobleaching. Data is presented $\pm$ SEM, $n=3$, where 5 fields, each containing at least 1 cell were imaged in three separate dishes on three separate occasions. Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

3.3.2 Effects of OXPHOS Inhibition on Mitochondrial Fusion in Differentiated PC12 cells

Previous studies have shown that inhibition of the electron transport chain has deleterious effects on the ability of the mitochondria to produce ATP and on a myriad of other cellular functions (Schon and Przedborski, 2011) (Liu et al., 2002). As a result, it was decided to investigate the effects of a number of potent inhibitors of the ETC and proton ionophores on mitochondrial fusion in differentiated PC12 cells. Dissipation of $\Delta\Psi_m$ using 5 μ M FCCP resulted in almost a complete halt of fusion, as shown in Fig 3.4, with a mean PI of 92.78% 45 min after photoactivation.

Inhibition of the various complexes of the ETC also resulted in a significant reduction in mitochondrial fusion in differentiated PC12 cells. Inhibition of CI with 10 μ M rotenone (Fig 3.5) or 2.4 μ M piericidin A (Fig 3.6) resulted in mean PIs of 92.5% and 95.6% respectively, indicating a significant reduction in mitochondrial fusion. CIII inhibition with 2.4 μ M antimycin A resulted in a significant reduction in PI with an average of 78.5% after 45 minutes (Fig 3.7). CIV was inhibited with 5mM KCN and this also resulted in a significant decrease in fusion with a mean PI of 87.3% 45 minutes after photoactivation (Fig 3.8). Finally, ATP synthase was inhibited using 2 μ g/ml oligomycin and as expected this resulted in a large decrease in mitochondrial fusion as shown by a mean PI of 84.1% after the experiment had been allowed to run its course (Fig 3.9). Taken together, these results suggest that differentiated PC12 cells are highly reliant on OXPHOS to produce the ATP required for a myriad of mitochondrial functions and inhibition of the correct function of this system results in severe deficits of mitochondrial fusion. Finally, as demonstrated in Fig 3.10, incubation with ETC inhibitors caused a fragmentation of the mitochondrial network 1 hour after treatment. The mitochondria appear to undergo a morphological change and become more rounded and punctate as well as displaying a decrease in fusion rates.

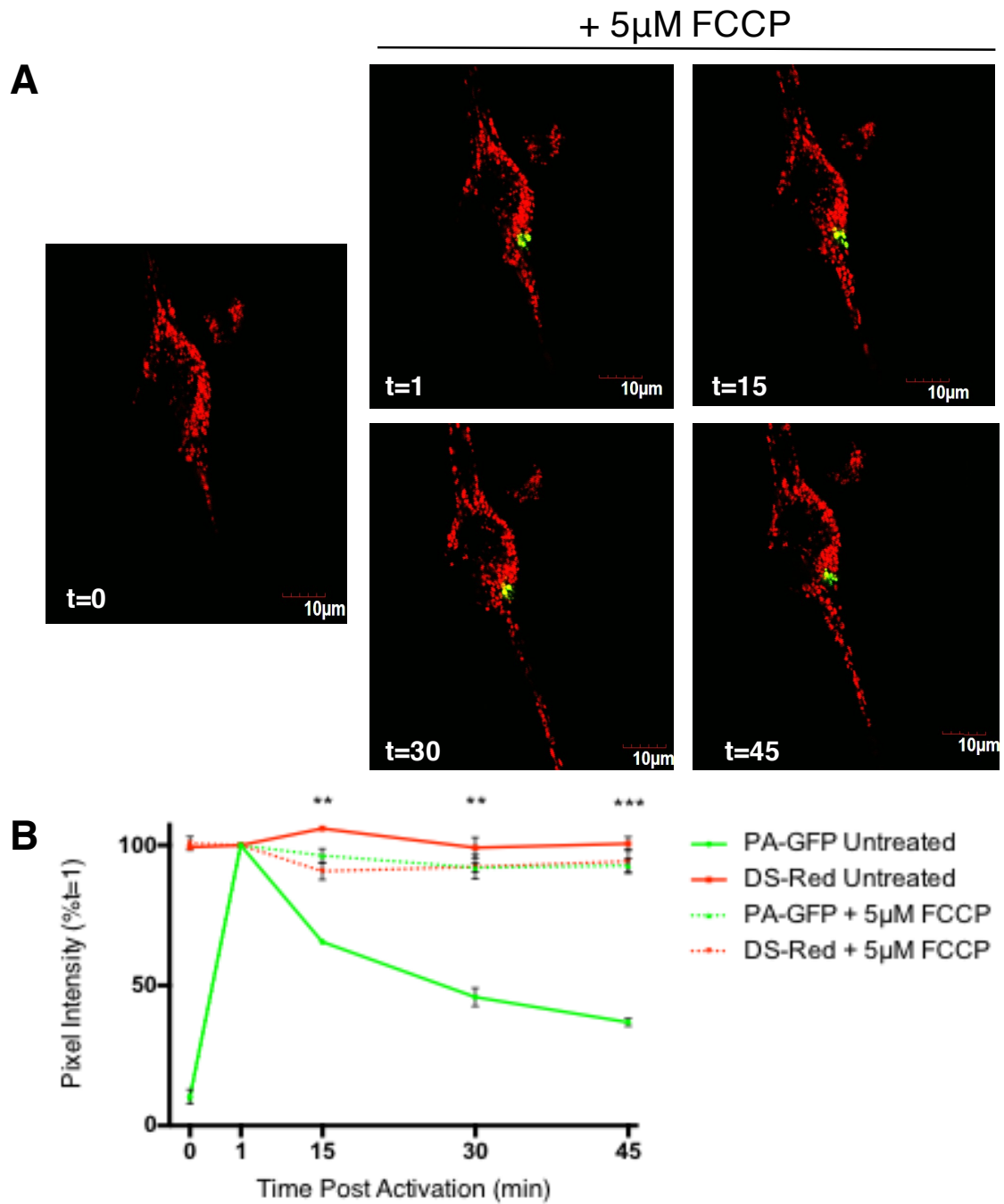


Figure 3.4: Dissipation of $\Delta\psi_m$ inhibits mitochondrial fusion in differentiated PC12 cells.

(A) Images representative of the effect of a 20 min pre-incubation with 5 μ M FCCP on mitochondrial fusion. (B) Quantitative data shows the inhibition of fusion in FCCP treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, n=3. Unpaired t-test at each time point with Welch correction for unequal variance. (Time points follow for all image positions in subsequent figures).

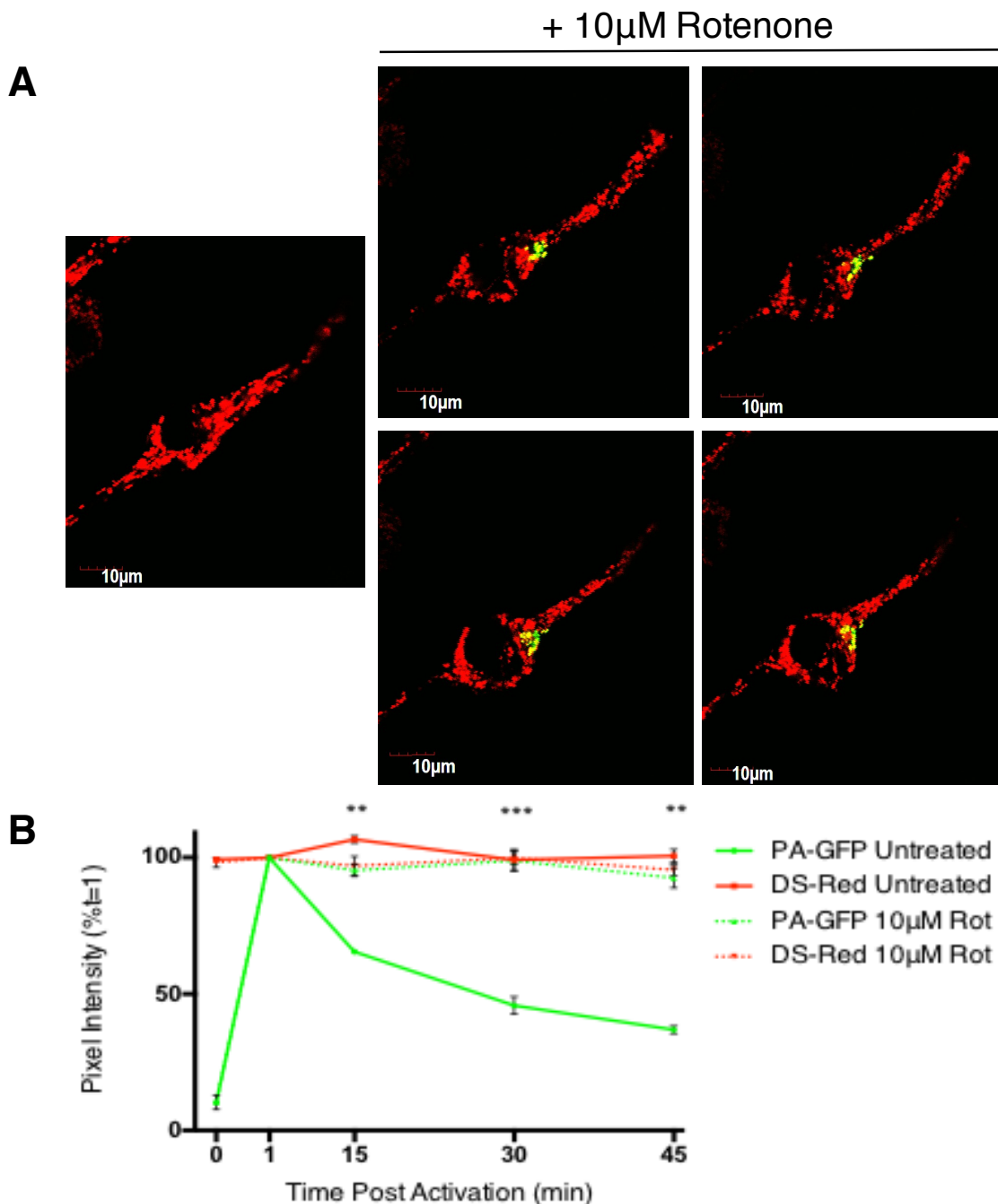


Figure 3.5: Inhibition of complex I with rotenone inhibits mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 20 min pre-incubation with 10 μ M rotenone on mitochondrial fusion. (B) Quantitative data shows the inhibition of fusion in rotenone treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, n=3. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

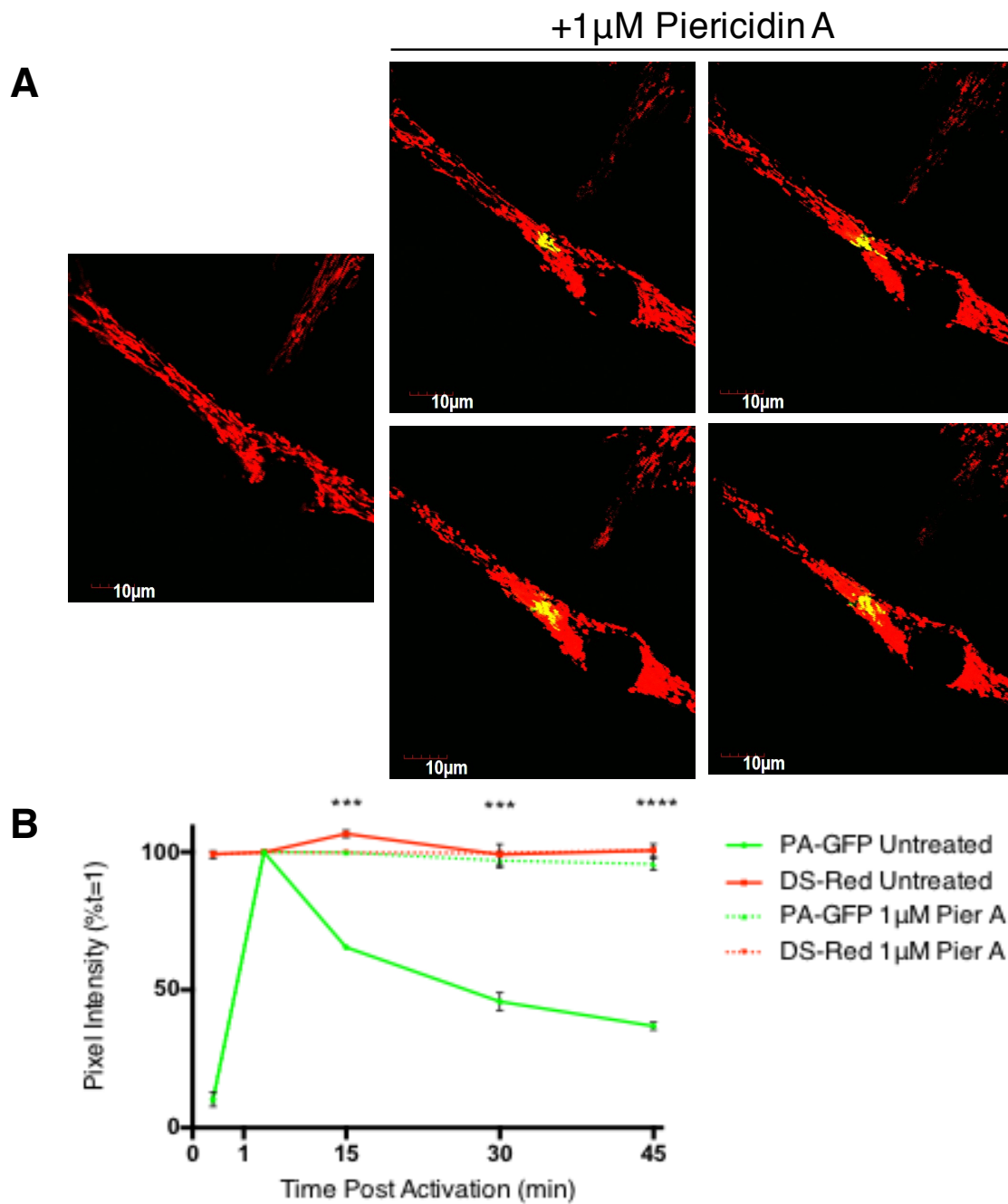


Figure 3.6: Inhibition of complex I with piericidin A inhibits mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 20 min pre-incubation with 1 μ M piericidin A on mitochondrial fusion. (B) Quantitative data shows the inhibition of fusion in piericidin A treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, n=3. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

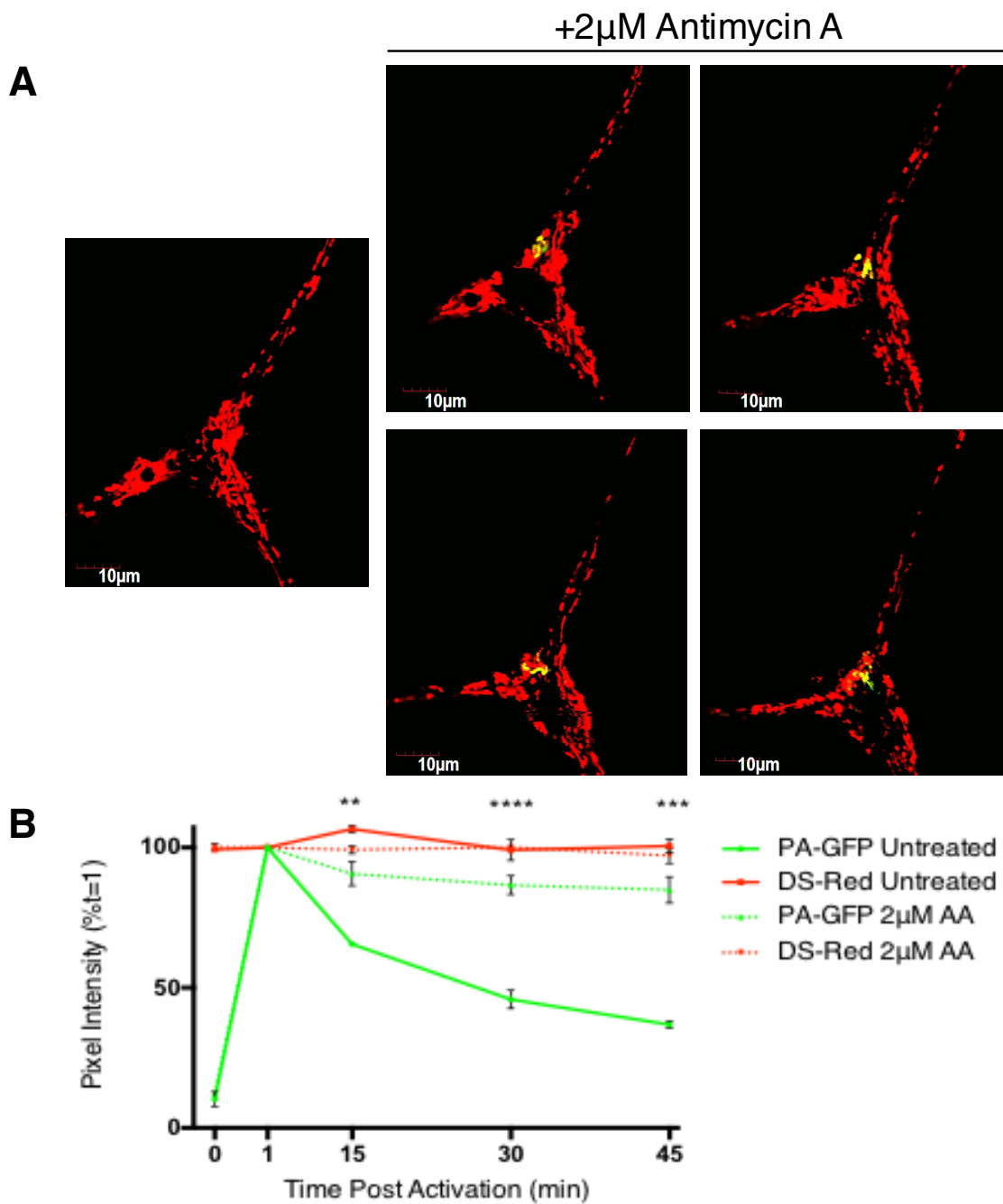


Figure 3.7: Inhibition of complex III with antimycin A inhibits mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 20 min pre-incubation with 2 μ M antimycin A on mitochondrial fusion. (B) Quantitative data shows the inhibition of fusion in antimycin A treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, n=3. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

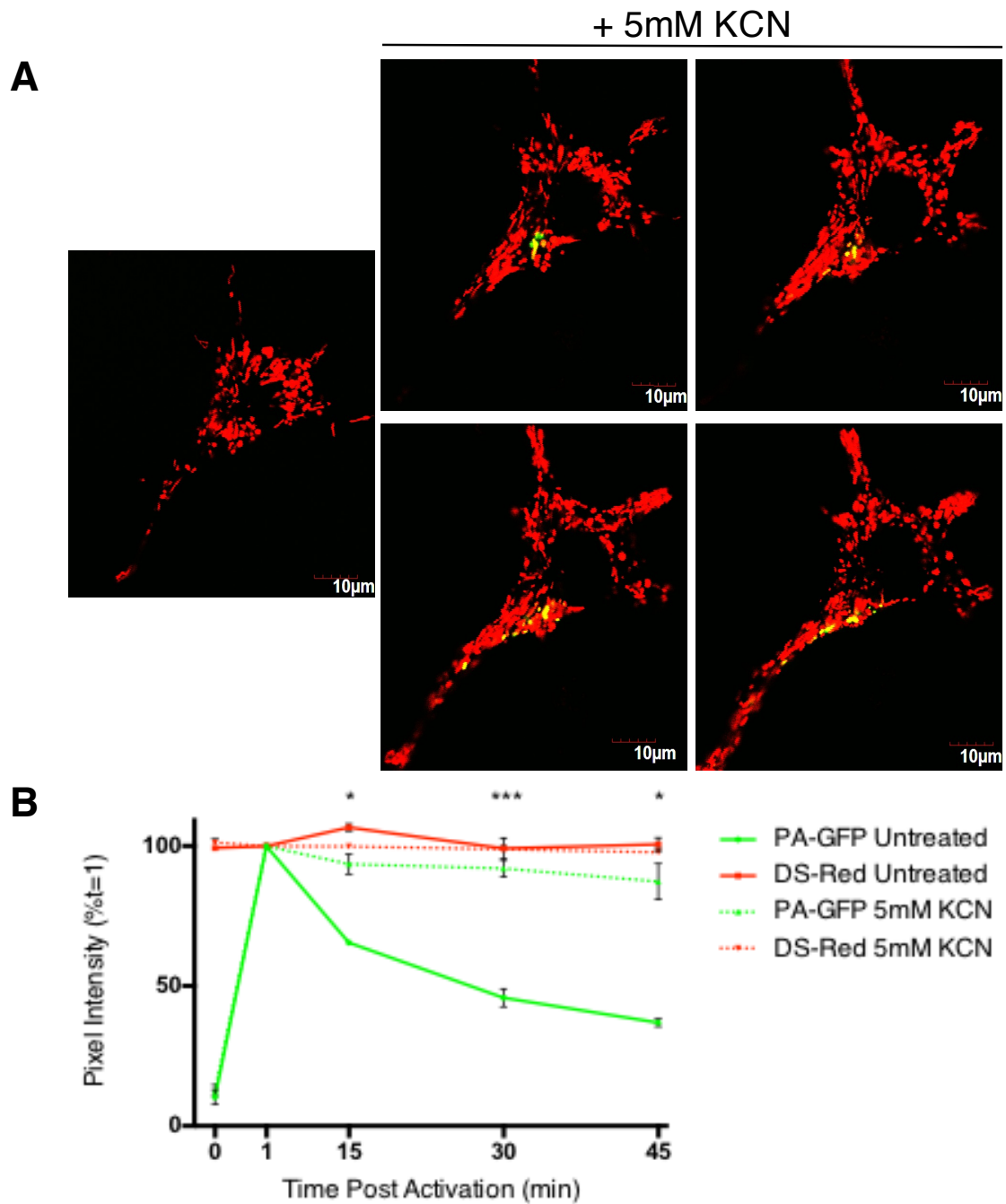


Figure 3.8: Inhibition of complex IV with potassium cyanide inhibits mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 20 min pre-incubation with 5 mM potassium cyanide on mitochondrial fusion. (B) Quantitative data shows the inhibition of fusion in potassium cyanide treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

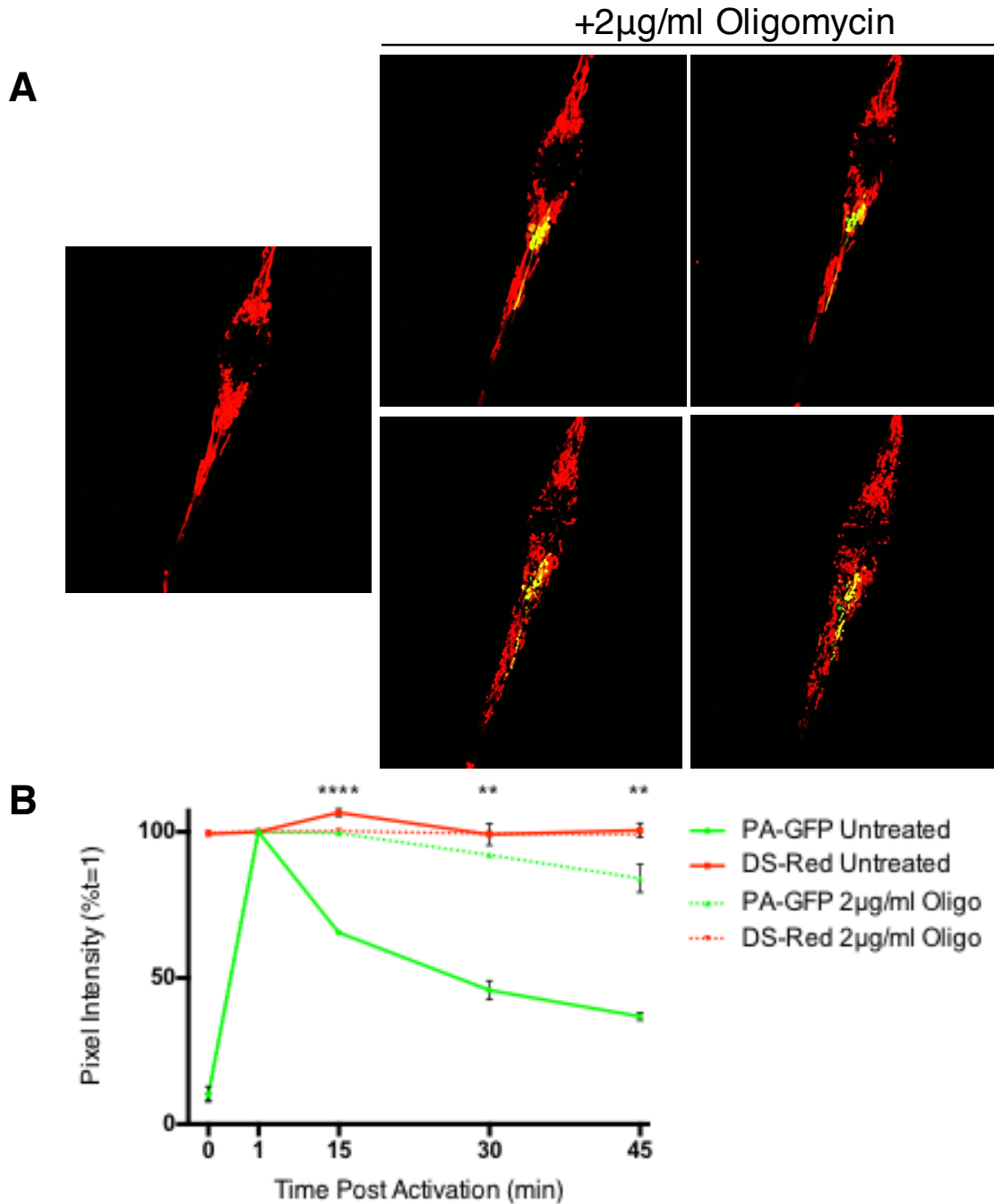


Figure 3.9: Inhibition of ATP synthase with oligomycin inhibits mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 20 min pre-incubation with 2 μ g/ml oligomycin on mitochondrial fusion. (B) Quantitative data shows the inhibition of fusion in oligomycin treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, n=3. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

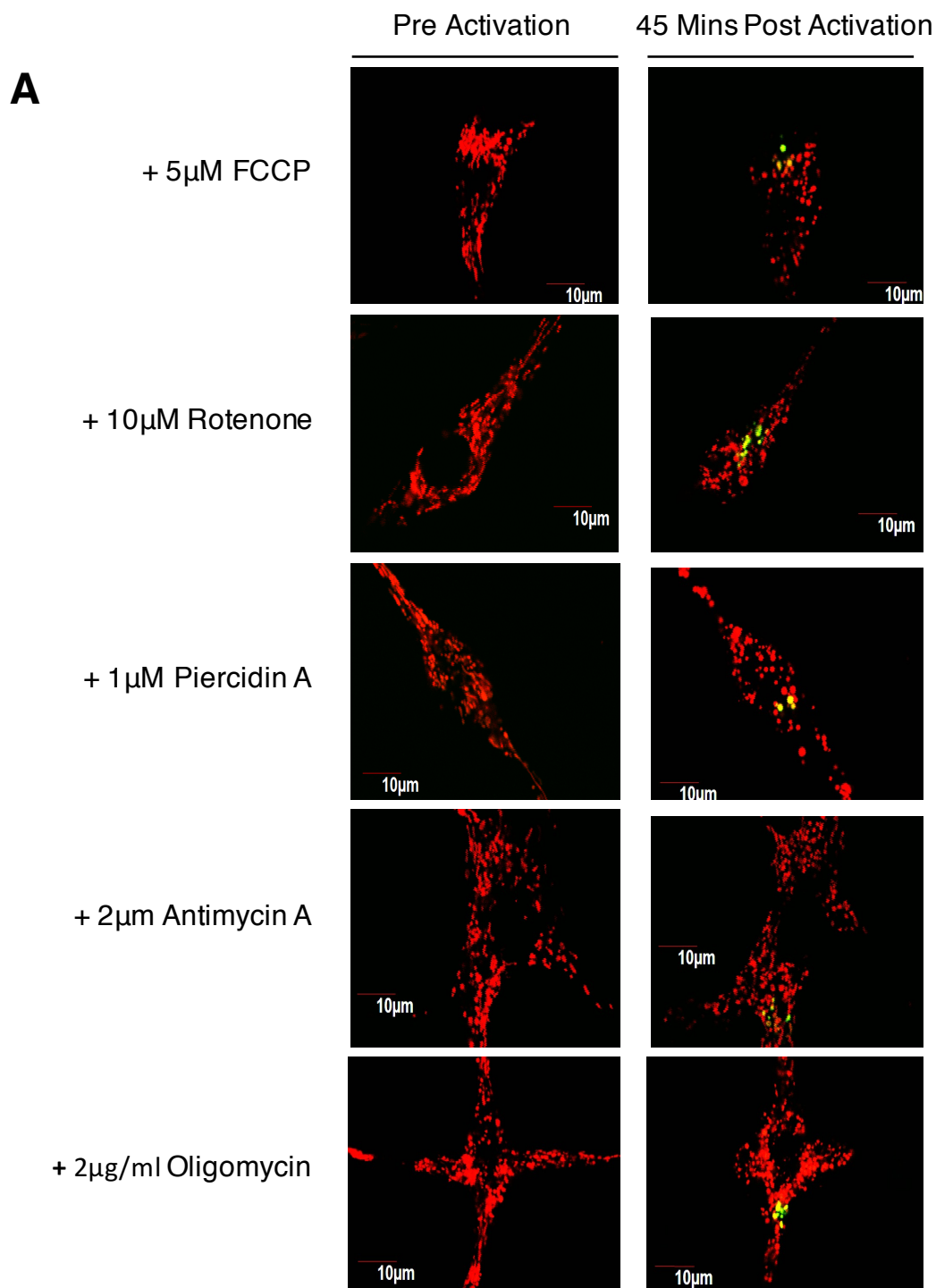


Figure 3.10: Dissipation of $\Delta\psi_m$ or inhibition of the ETC causes fragmentation of the mitochondrial network in differentiated PC12 cells. The left panel shows images taken pre-photo-activation, after 10-15 min treatment with inhibitors, where there is little fragmentation visible. In the right panel, taken 45 min post photo-activation, and 1 hour post inhibitor treatment, mitochondria appear to become more rounded and punctate showing a fragmentation of the mitochondrial network.

3.3.3 Effects of OXPHOS Inhibition on $\Delta\Psi_m$ in Differentiated PC12 cells

Given the strong inhibition of mitochondrial fusion observed after the addition of ETC inhibitors, we decided to test the effect of these inhibitors on $\Delta\Psi_m$. Based on the hypothesis that mitochondria with a lower $\Delta\Psi_m$ after fission are prepared for degradation, whereas those with a higher $\Delta\Psi_m$ are trafficked to sites with a high energy demand and participate in further fission events (Gilad et al., 2008), we expected to see a significant changes in $\Delta\Psi_m$ correlating with the decreased fusion rates. Mitochondrial membrane potential was measured using microscopic measurement of TMRM fluorescence. TMRM is a cationic, red-orange fluorescence emitting dye sequestered by mitochondria in $\Delta\Psi_m$ -dependent manner. At non-quenching concentrations, mitochondrial depolarization is indicated by loss of signal (and conversely, hyperpolarization by increase in signal). To confirm the sensitivity of this method in detecting $\Delta\Psi_m$ changes, time course experiments with FCCP were conducted. As expected, dissipation of $\Delta\Psi_m$ using FCCP resulted in a complete decrease in PI to 9.2% after only 1 min, which was maintained throughout the course of imaging as can be seen in Fig 3.11. Exposure of differentiated PC12 cells to CI and CIII inhibitors led to a significant PI decreases after only 1 minute, while 20 minutes after administration of inhibitors mean PI had decreased to 6.7% and 5.8% respectively. Inhibition of complex IV resulted in a more modest decrease in $\Delta\Psi_m$ which was still significantly different at 20 min post addition of KCN. Inhibition of ATP synthase resulted in a hyperpolarisation of $\Delta\Psi_m$ where a significant increase of 21.64% over baseline levels was observed over a 20 min treatment with oligomycin as seen in Fig 3.13. These results demonstrate that differentiated PC12 cells are unable to maintain fusion rates when there is a significant reduction in $\Delta\Psi_m$ or where ATP synthesis is blocked.

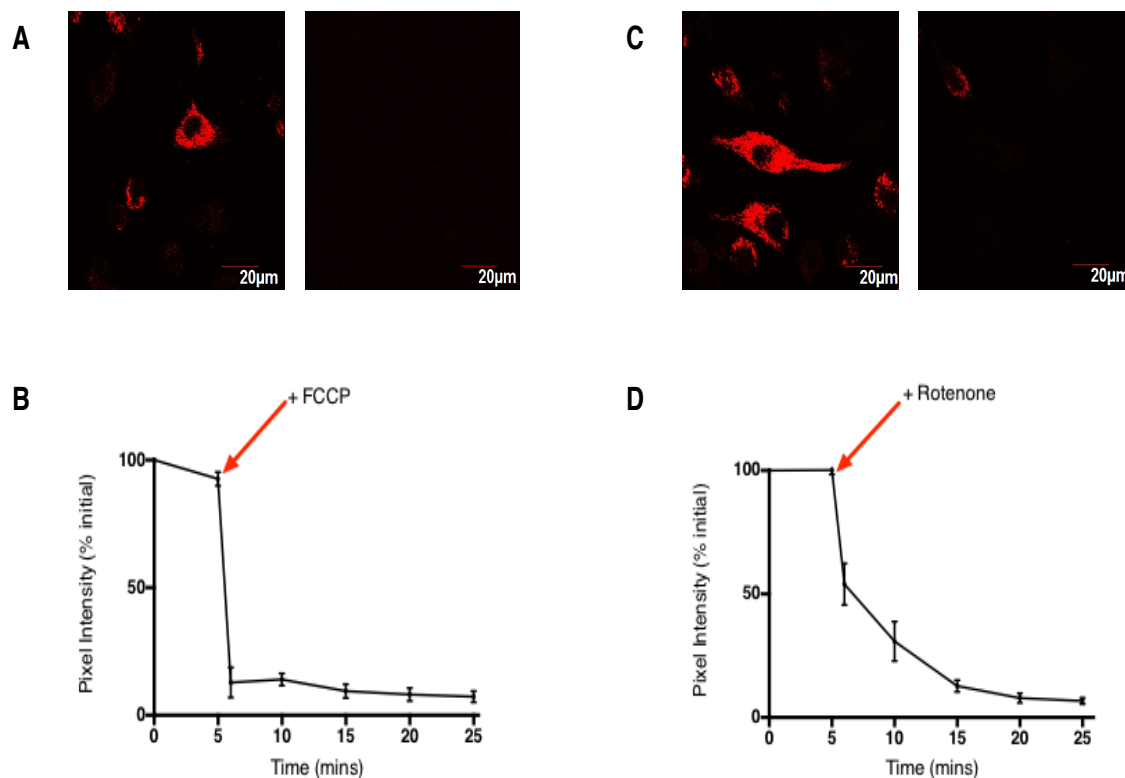


Figure 3.11: Addition of FCCP and inhibition of complex I dissipates $\Delta\psi_m$ in differentiated PC12 cells. (A) and (C) Images representative of TMRM fluorescence before (left image) and after (right image) the addition of 5 μ M FCCP (A) or 10 μ M rotenone (C). (B) Quantitative data shows the addition of an ionophore after 5 min results in an immediate and sustained depolarization of mitochondria. (D) Quantitative data shows the addition of a complex I inhibitor after 5 min results in an immediate decrease in $\Delta\psi_m$ which is further reduced until complete depolarization 20 min after the addition of rotenone. Data presented as mean $\pm$ SEM, $n=3$ and expressed as % initial (pre-treatment) values.

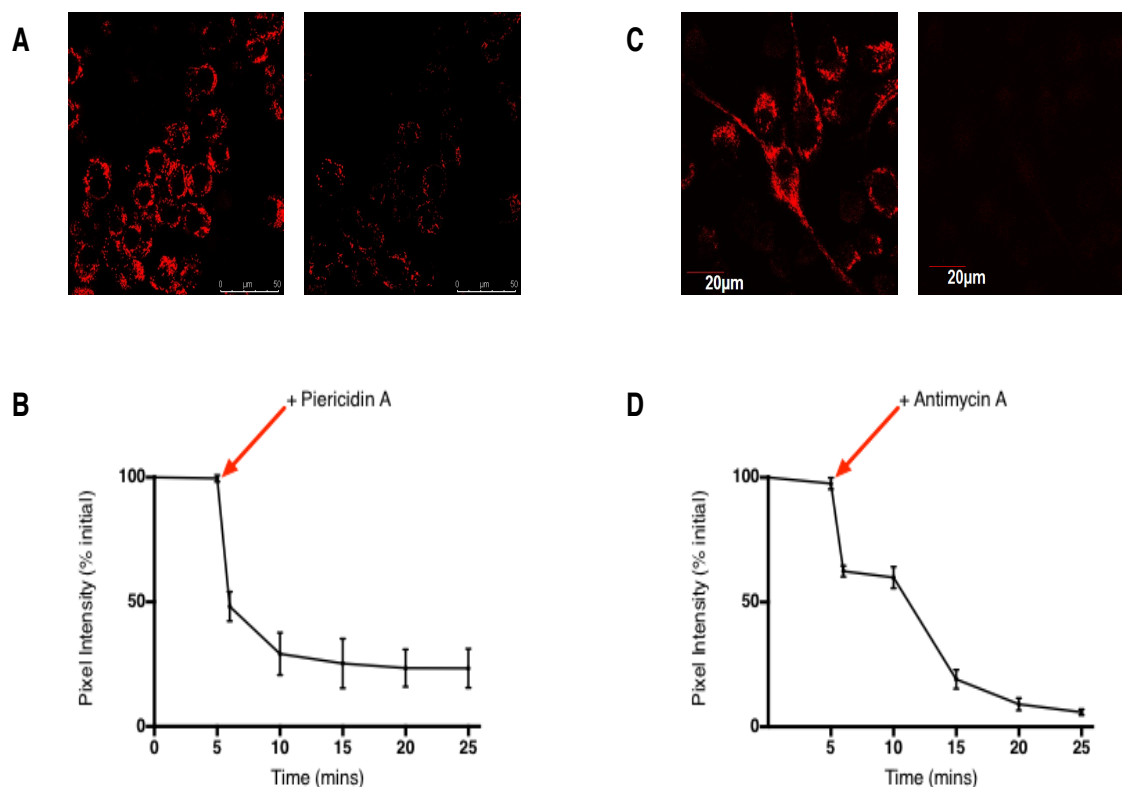


Figure 3.12: Addition of piericidin A or antimycin A dissipates $\Delta\psi_m$ in differentiated PC12 cells. (A) and (C) Images representative of TMRM fluorescence before (left image) and after (right image) the addition of 1 μM piericidin A (A) or 2 μM antimycin A (C). (B) Quantitative data shows the addition of a complex I inhibitor after 5 min results in an immediate decrease in $\Delta\psi_m$ which is further reduced until complete depolarization 20 min after the addition of piericidin A. (D) Quantitative data shows the addition of a complex III inhibitor after 5 min results in an immediate decrease in $\Delta\psi_m$ which is further reduced until complete depolarization 20 min after the addition of antimycin A. Data presented as mean $\pm$ SEM, $n=3$ and expressed as % initial (pre-treatment) values.

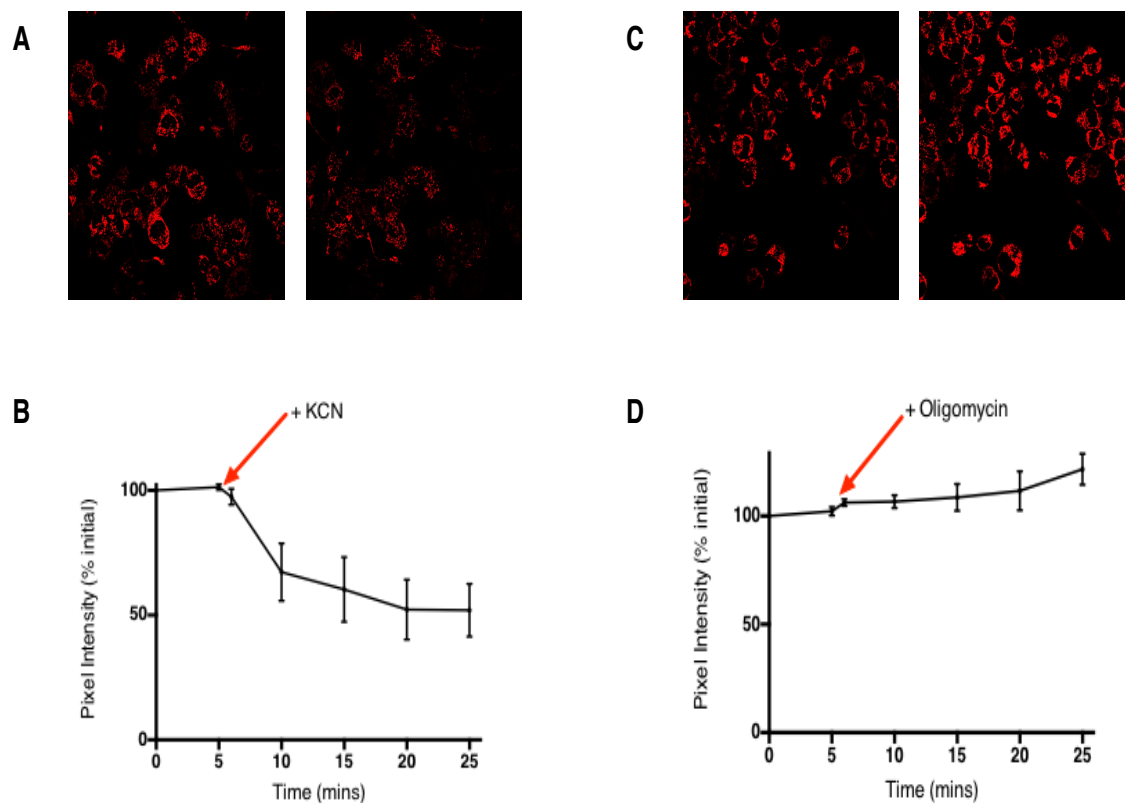


Figure 3.13: Addition of potassium cyanide significantly depolarizes mitochondria and addition of oligomycin significantly hyperpolarizes mitochondria in differentiated PC12 cells. (A) and (C) Images representative of TMRM fluorescence before (left image) and after (right image) the addition of 5 mM potassium cyanide (A) or 2 μg/ml oligomycin (C). (B) Quantitative data shows the addition of a complex IV inhibitor after 5 min results in a gradual decrease in $\Delta\psi_m$ which continues until significant depolarization occurs 10 min after the addition of potassium cyanide. (D) Quantitative data shows the addition of an ATP synthase inhibitor after 5 min results in a gradual increase in $\Delta\psi_m$ which continues until significant depolarization occurs 20 min after the addition of oligomycin. Data presented as mean $\pm$ SEM, n=3 and expressed as % initial (pre-treatment) values.

A

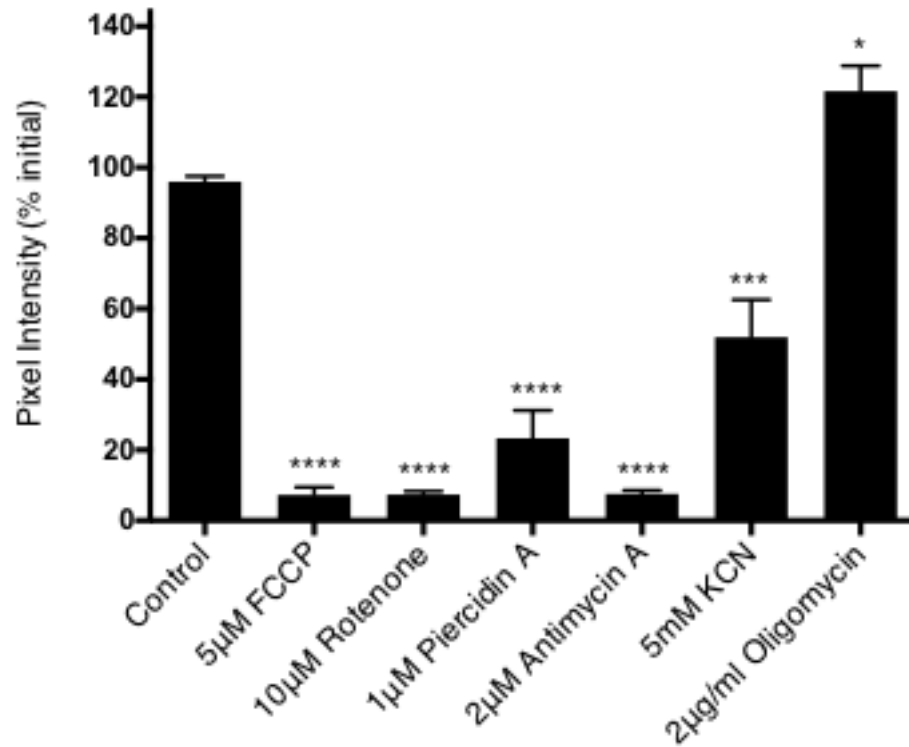


Figure 3.14: Inhibition of OXPHOS results in significant changes in $\Delta\psi_m$ in differentiated PC12 cells. Microscopic measurement of TMRM fluorescence showed that differentiated PC12 cells are significantly depolarized following 20 min treatment with an ionophore or inhibition of complexes I, III or IV and significantly hyperpolarized following inhibition of ATP synthase for 20 min. Data presented as mean $\pm$ SEM (n=3) and expressed as % initial (pre-treatment) values. An ordinary one-way ANOVA was performed followed by Dunnett's multiple comparison test. Data presented as mean $\pm$ SEM, n=3. Results which were significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

3.3.4 Effects of OXPHOS Inhibition on ROS Production in Differentiated PC12 Cells and Primary Cortical Neurons

It has been widely reported the inhibition of the ETC results in an increase in ROS production, of which Superoxide ($O_2^{\bullet-}$) is the proximal ROS. In particular, inhibition of CI and CIII have been shown to cause large increases in ROS *in vitro* (Murphy, 2009) (Liu et al., 2002). As a result, we decided to test the effects of high concentrations of ETC inhibitors on ROS production in differentiated PC12 cells. 2',7'-Dichlorohydrofluorescein diacetate (H_2DCFDA) was used to measure ROS production using a plate reader. H_2DCFDA detects a wide range of ROS as it freely permeates the plasma membrane and is hydrolysed in the cytosol to form the DCFH carboxylate anion. Oxidation results in the formation of fluorescent DCF which is maximally excited at 495 nm and emits as 520 nm (Kalyanaraman et al., 2012). This oxidation is a two-step process, where the DCF radical is first formed and then it is further oxidized to DCF in a reaction with molecular oxygen. The first oxidation reaction can be catalysed by several different radical species including hydroxyl radical, carbonate radical and nitrogen dioxide indicating the wide variety of ROS that this probe can detect. Despite its reputation as a hydrogen peroxide detecting probe, H_2DCFDA does not interact with H_2O_2 directly but requires the presence of peroxidases or other enzymes containing transition metals. Indeed, an alteration of the signal can be caused by antioxidant enzymes, such as superoxide dismutase, or superoxide that competes with the probe for the ROS (Winterbourn, 2014). Cells were loaded with $5\mu M$ H_2DCFDA in Krebs buffer and the rate of ROS production as measured by an increase in fluorescence was observed over 1 hour.

Interestingly, only inhibition of CIII with antimycin A caused a significant increase in the rate of ROS production as can be seen in Fig 3.15. Inhibition of the other components of the ETC had slight but non-significant effects. It should be noted as mentioned above that this probe has some limitations as $O_2^{\bullet-}$ and other ROS may already be scavenged by the cells innate antioxidant system before getting the opportunity to oxidize H_2DCF and thus increase the level of fluorescence detected in the plate reader.

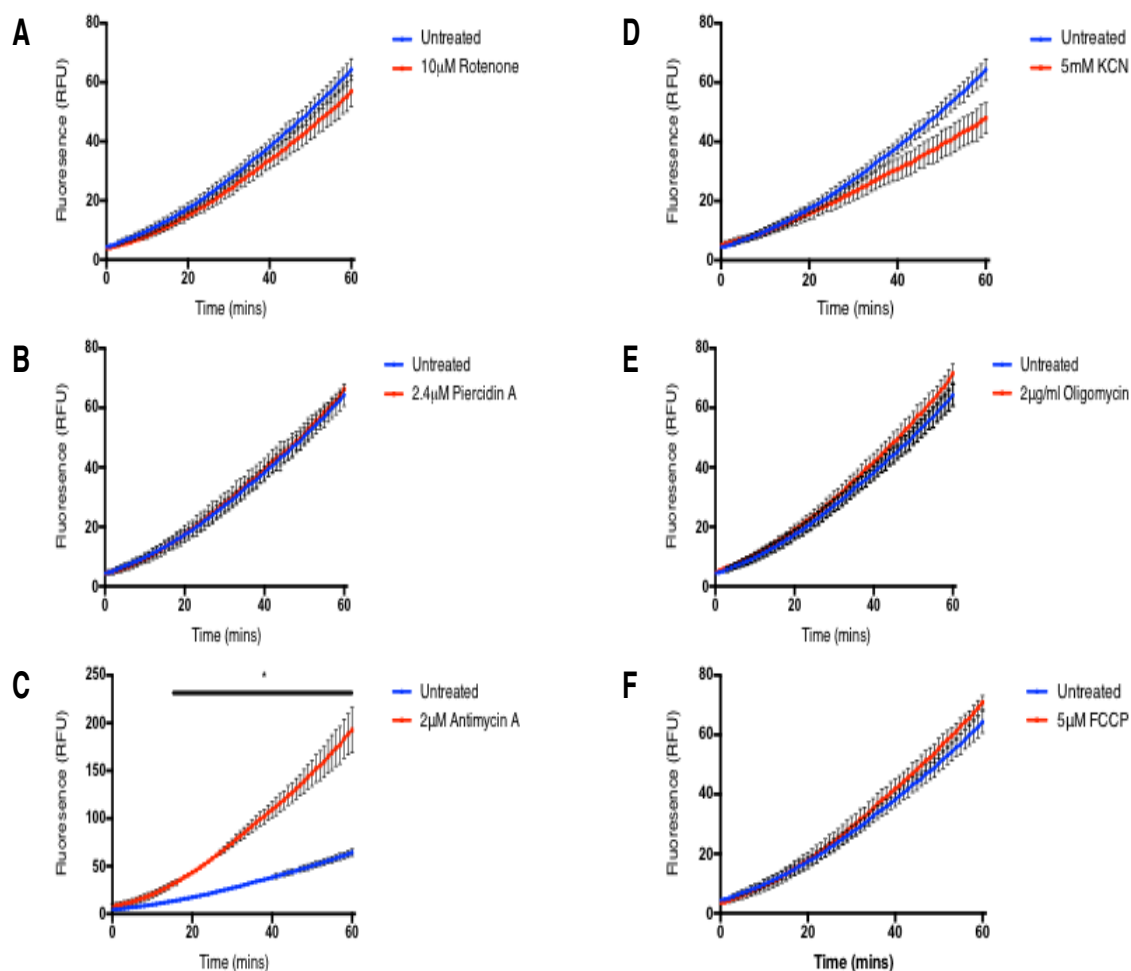


Figure 3.15: Inhibition of CIII results in an increase in ROS production in differentiated PC12 cells. Cells were differentiated in clear-bottomed black 96 well plates for 48 hours before being washed twice and incubated with 5µM H₂DCFDA in krebs buffer and assayed in a SpectraMax PLUS microplate spectrophotometer. Cells were incubated with H₂DCFDA in the presence of 10 µM rotenone (A), 1 µM piericidin A (B), 2 µM antimycin A (C), 5 mM KCN (D), 2 µg/ml oligomycin (E) or 5 µM FCCP (F) and compared to control wells loaded with battery. Data presented as mean ± SEM, n=3. An individual t-test was performed at each time point and values that are significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

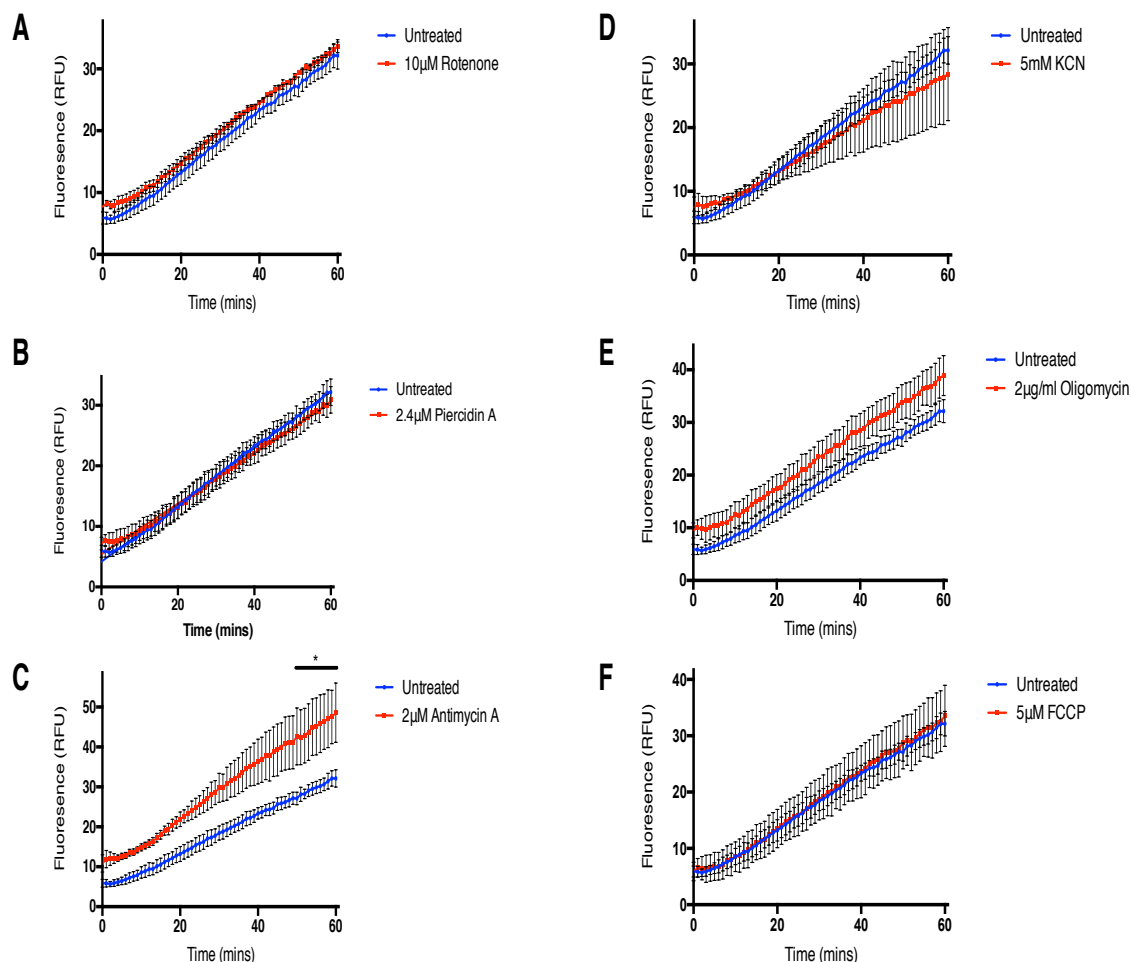


Figure 3.16: Inhibition of CIII results in an increase in ROS production in primary cortical neurons. Cells were differentiated in clear-bottomed black 96 well plates for 8 days before being washed twice and incubated with 5 μ M H₂DCFDA in krebs buffer and assayed in a SpectraMax PLUS microplate spectrophotometer. Cells were incubated with H₂DCFDA in the presence of 10 μ M rotenone (A), 2.4 μ M piericidin A (B), 2 μ M antimycin A (C), 5 mM KCN (D), 2 μ g/ml oligomycin (E) or 5 μ M FCCP (F) and compared to control wells loaded with battery. Data presented as mean $\pm$ SEM, n=3. An individual t-test was performed at each time point and values that are significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

3.3.5 Effect of Inhibition of OXPHOS on Mitodynamin Expression in Differentiated PC12 cells

As discussed in section 1.3, mitochondrial dynamics can be regulated through post translational modification of mitodynamins, including phosphorylation, ubiquitination, O-GlcNAcylation and proteolytic cleavage. To assess whether the observed decrease in fusion upon OXPHOS inhibition is due to PTM of mitodynamins, western blots for fusion mediators Opa1, Mfn1 and Mfn2 and fission mediator Drp1 were performed on whole cell lysates from differentiated PC12 cells treated for 20 mins with either 10 μ M rotenone, 1 μ M piericdin A, 2 μ M antimycin A, 5 mM potassium cyanide, 2 μ g/ml oligomycin or 5 μ M FCCP.

With a 20 min treatment, there was significant changes in the pattern and level of expression of Opa1, specifically a cleavage of L-Opa1 to S-Opa1 as indicated by the changes in the ratio of L-Opa1 to S-Opa1 in Fig 3.17. It has previously been shown that dissipation of $\Delta\psi_m$ promotes a cleavage of Opa1 to short isoforms (Ishihara et al., 2006, Guillery et al., 2008) which inhibits mitochondrial fusion and this study confirms that as cells treated with FCCP show an almost complete loss of L-Opa1 and a large decrease in the ratio as compared to untreated cells. Baker et al. (2014) showed that treatment with oligomycin stimulated Opa1 processing, although not as efficiently as seen with complete $\Delta\psi_m$ membrane dissipation. Interestingly, in the same paper, treatment with antimycin A resulted in a loss of $\Delta\psi_m$ but L-Opa1 remained stable indicating the sensitivity of Opa1 to a variety of cellular stress responses, and not just $\Delta\psi_m$. However, it should be noted the concentration of antimycin A was lower than that used here and these experiments were carried out in MEFs which may be less susceptible to ETC inhibition than a neuronal model such as differentiated PC12 cells. There have been mixed reports as regards Opa1 processing after complex I inhibition as Peng et al. (2016) have shown a dose dependent decrease in both forms of Opa1 after rotenone treatment while others have shown no processing occurring after rotenone treatment (Li et al., 2017, Guillery et al., 2008). These results highlight the varying susceptibility of different cell types to ETC inhibition in terms of

mitochondrial fusion dynamics. Here we report a significant decrease in the ratio of L-Opa1 to S-Opa1 in non-fusing mitochondria indicating this shift is at least partly responsible for the abolition of mitochondrial fusion. Finally, we show that 20 min treatment with 5 mM KCN also causes an increase in S-Opa1 as a percentage of the total amount of Opa-1 expressed in PC12 cell lysates indicating a possible role for Opa-1 cleavage in the abolition of fusion seen in cells where CIV is inhibited.

No significant changes were seen in Mfn1/2 protein expression after treatment with any of the inhibitors which is largely in agreement with much of the published literature on the topic. Interestingly, there were also no significant changes in Drp1 expression after treatment with OXPHOS inhibitors. This is in contrast to a number of previously published papers which show a sustained decrease in Drp1 levels after such treatments which is accompanied by increases in phosphorylated Drp1 (pDrp1) (Cereghetti and Stangherlin, 2008, Peng et al., 2016). However, the antibody used in this study does not detect phosphorylated Drp1 and it is a measure of whole cell lysate Drp1 whereas the studies mentioned above measure mitochondrial Drp1 as it is recruited to the mitochondria and then phosphorylated in order to promote fission. Further studies will be required to determine any alterations in the levels of phosphorylation of Drp1 in differentiated PC12 cells after ETC inhibition.

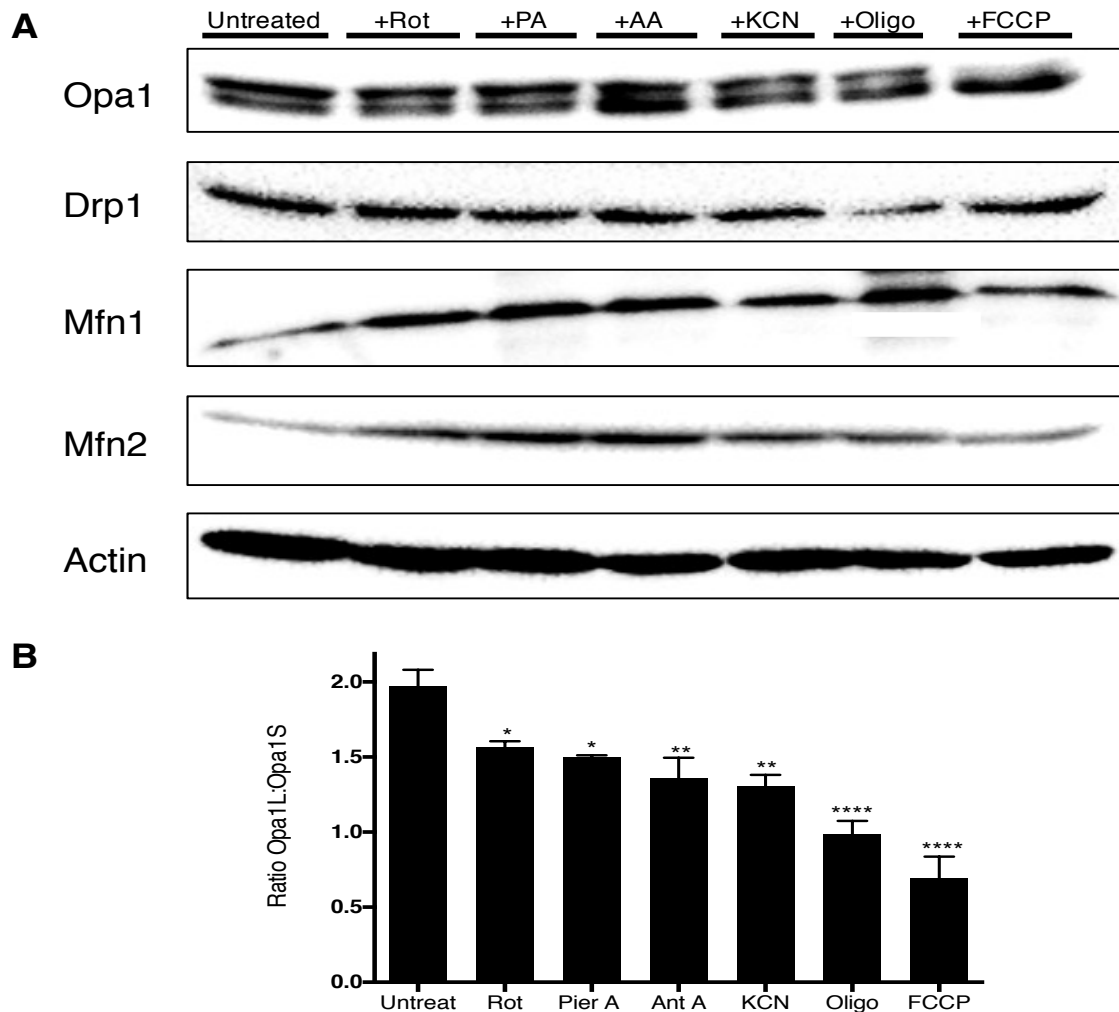


Figure 3.17: Expression of the fusion/fission machinery remains unchanged except for alterations in Opa1 isoforms in differentiated PC12 cells treated with OXPHOS inhibitors.

(A) PC12 cells were differentiated for 48 hours and treated with vehicle (untreat), 10 μ M rotenone (rot), 1 μ M piericidin A (pier A), 2 μ M antimycin A (anti A), 5 mM potassium cyanide (KCN), 2 μ g/ml oligomycin (oligo) and 5 μ M FCCP (FCCP) for 20 min before being lysed with RIPA buffer and analysed for fusion mediating mitodynaminins by western blot. No significant changes were observed in protein levels of Drp1, Mfn1 or Mfn2 but changes in the long and short forms of Opa1 were observed. Images representative of $n=3$. (B) Densitometry analysis of Opa1 western blots shows significant changes in the ratio of the long form: short form. In control samples, there is twice as much of the long form as the short form but upon in non-fusing mitochondria treated with OXPHOS inhibitors the ratio decreases to below 1.5 and there is an excess of short form over long form in those cells treated with FCCP. Ordinary one-way ANOVA followed by Dunnett's post comparison test. Data presented as mean $\pm$ SEM, $n=3$. Results which were significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

3.3.6 Threshold Effect of Complex I Inhibition on Mitochondrial Fusion in Primary Cortical Neurons

Given the sensitivity of mitochondrial fusion to OXPHOS inhibition in PC12 cells and neuronal cells (from a previous study in the lab), it was decided to investigate if there was a threshold of inhibition beyond which mitochondrial fusion was adversely affected by inhibition of complex I. Complex I was selected as previous work in the lab had determined that such thresholds exist in relation to oxygen consumption and ATP synthesis in synaptosomes and NSM (Pathak and Davey, 2008) and the fact that Ci inhibition has a widely acknowledged role in the onset of PD as previously discussed (Schapira et al., 1990). Piericidin A was chosen a selective inhibitor of complex I as previous experiments had shown that rotenone interferes with microtubule assembly and subsequently mitochondrial transport which may negatively affect fusion rates. In addition to this, it had been shown that 5 min incubation with 100 nM piericidin A was sufficient to completely inhibit mitochondrial fusion and induce fragmentation of the mitochondrial network by 45 min post activation so this was used as the minimum incubation time for all subsequent experiments. Otherwise, the same settings as had been used for measuring mitochondrial fusion in differentiated PC12 cells were used.

Thus, piericidin A was used to selectively inhibit complex I at concentrations ranging from 10 nM – 1 μ M. Five min exposure of neurons to 1 μ M, 50 nM and 20 nM resulted in a complete cessation of mitochondrial fusion and led to a fragmentation of the mitochondrial network at 45 min post photo-activation of the 6.25 μ m² ROI (previous results indicated complete cessation of fusion at 100 nM and 500 nM piericidin A). Neurons exposed to 10 nM or 15 nM piericidin A for 5 min showed fusion rates which were comparable to those of control cells and displayed no signs of mitochondrial fragmentation while those treated with 17.5 nM piericidin A showed slight but non-significant reductions in mitochondrial fusion and no evidence of mitochondrial fragmentation. These results suggest that primary cortical neurons can withstand a certain level of inhibition of complex I before mitochondrial fusion is affected.

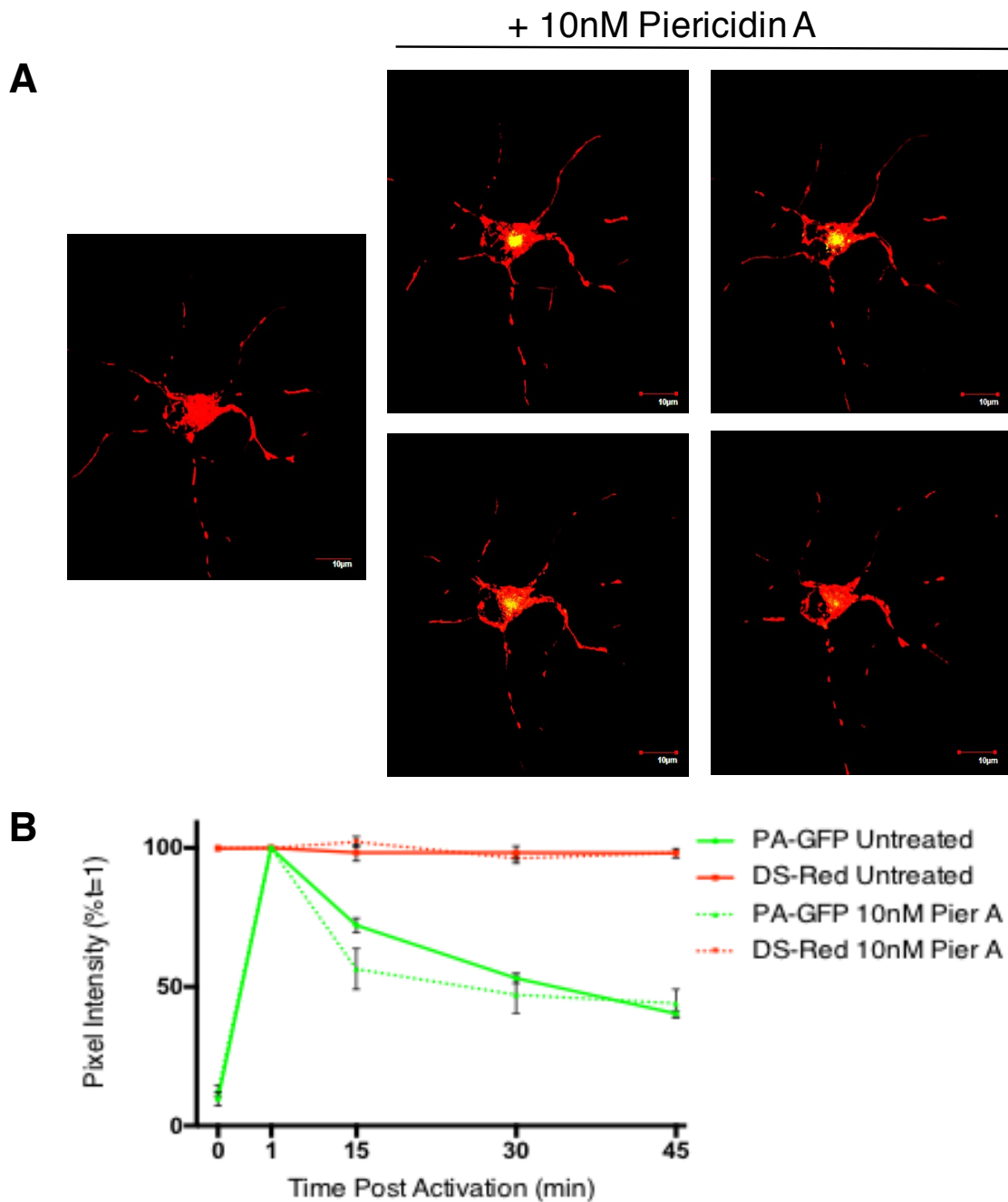


Figure 3.18: Inhibition of complex I with 10 nM piericidin A has no effect on mitochondrial fusion in primary cortical neurons. (A) Images representative of the effect of a 5 min pre-incubation with 10 nM piericidin A on mitochondrial fusion. (B) Quantitative data shows no inhibition of fusion in piericidin A treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

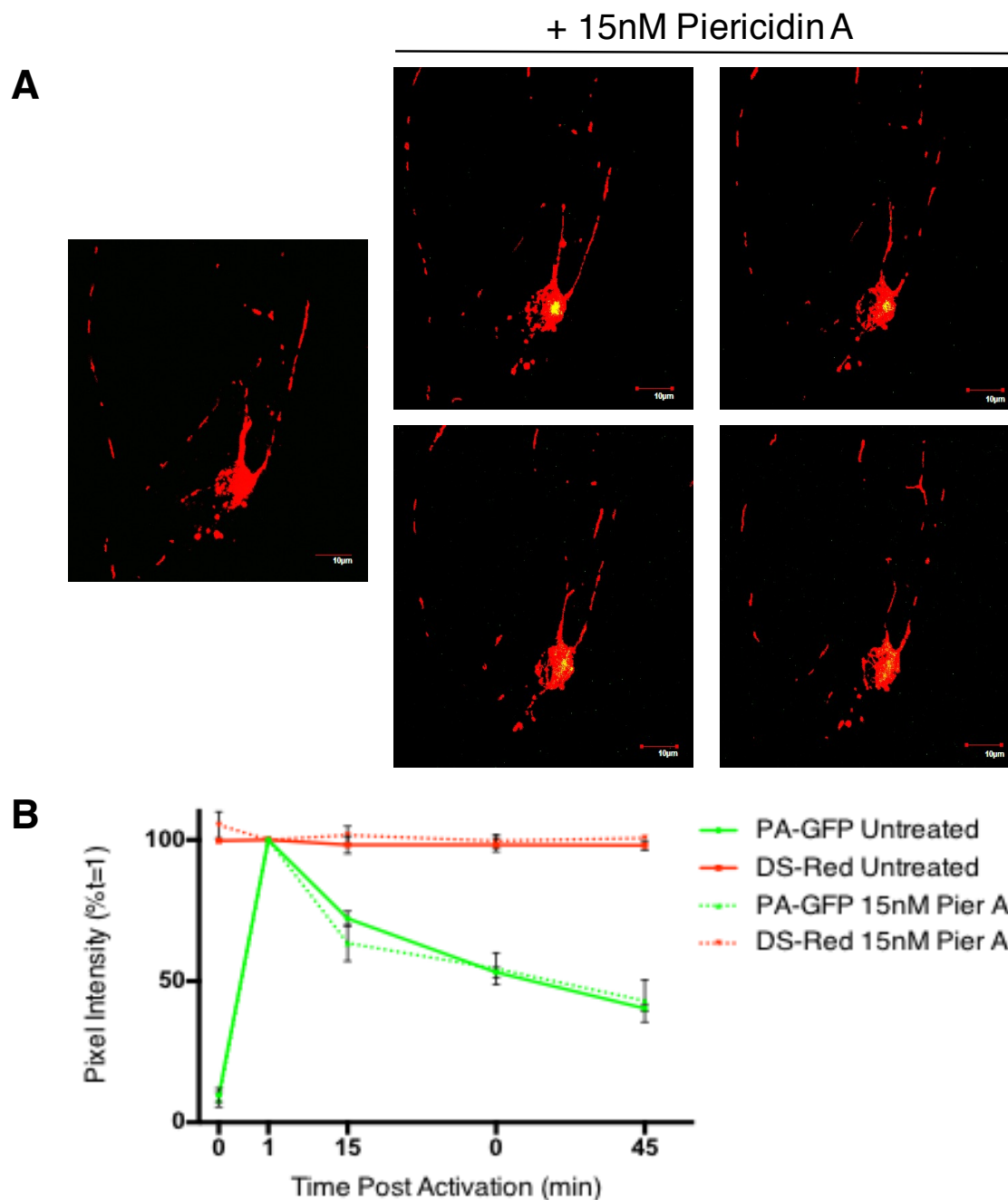


Figure 3.19: Inhibition of complex I with 15 nM piericidin A has no effect on mitochondrial fusion in primary cortical neurons. (A) Images representative of the effect of a 5 min pre-incubation with 15 nM piericidin A on mitochondrial fusion. (B) Quantitative data shows no inhibition of fusion in piericidin A treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

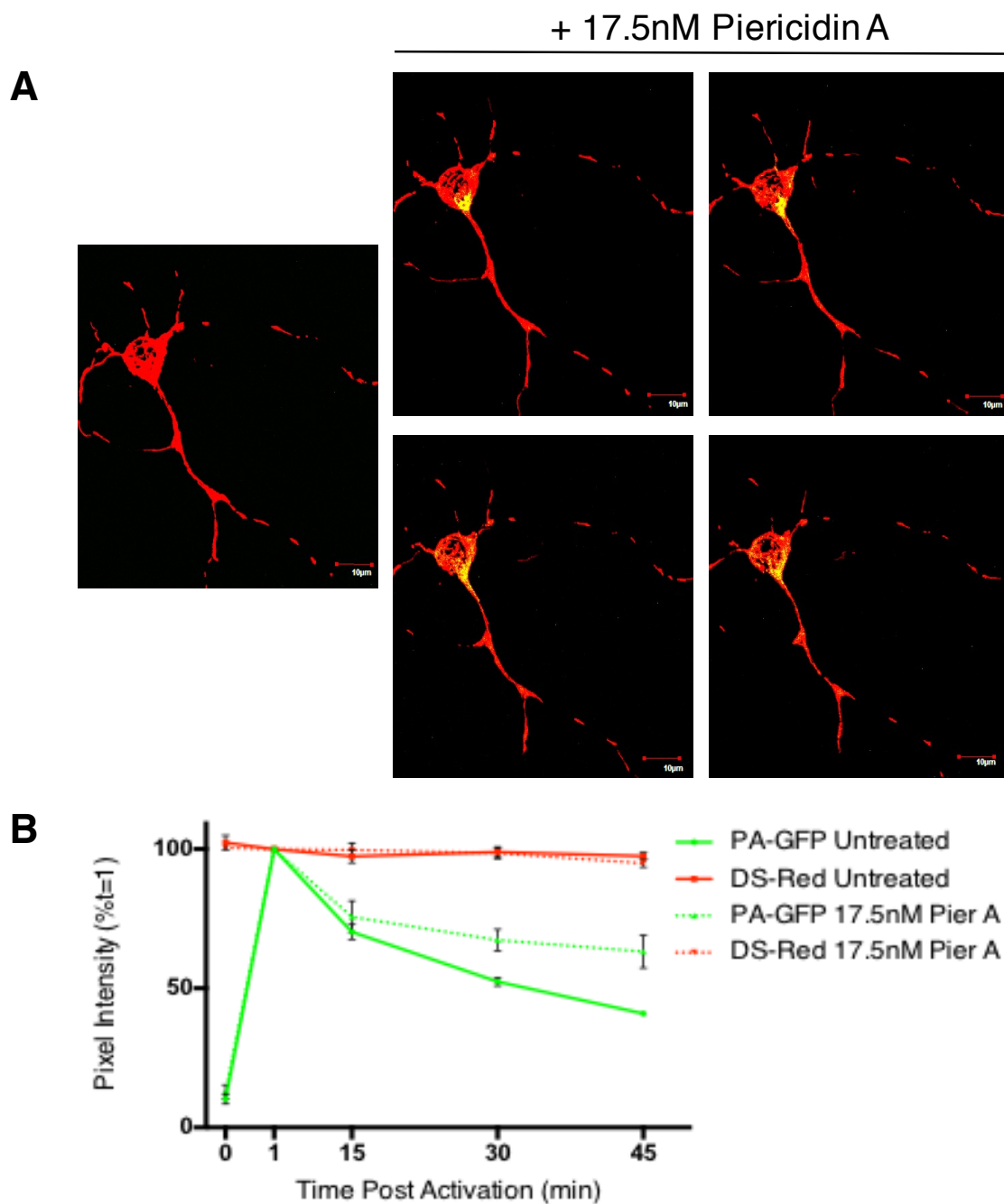


Figure 3.20: Inhibition of complex I with 17.5 nM piericidin A has a slight but non-significant effect on mitochondrial fusion in primary cortical neurons. (A) Images representative of the effect of a 5 min pre-incubation with 17.5 nM piericidin A on mitochondrial fusion. (B) Quantitative data shows no significant inhibition of fusion in piericidin A treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

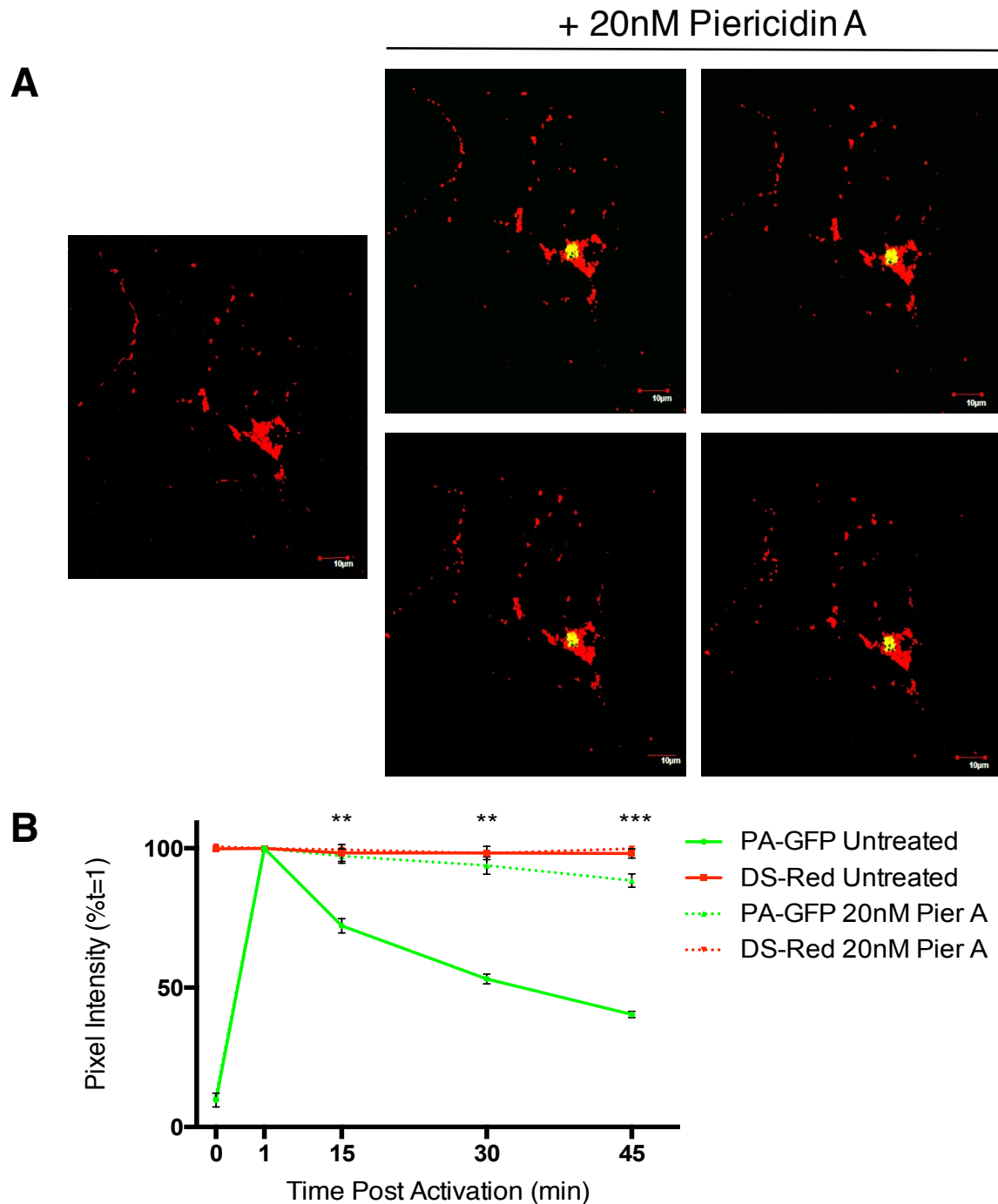


Figure 3.21: Inhibition of complex I with 20 nM piericidin A reduces mitochondrial fusion in primary cortical neurons. (A) Images representative of the effect of a 5 min pre-incubation with 20 nM piericidin A on mitochondrial fusion. (B) Quantitative data shows significant inhibition of fusion in piericidin A treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

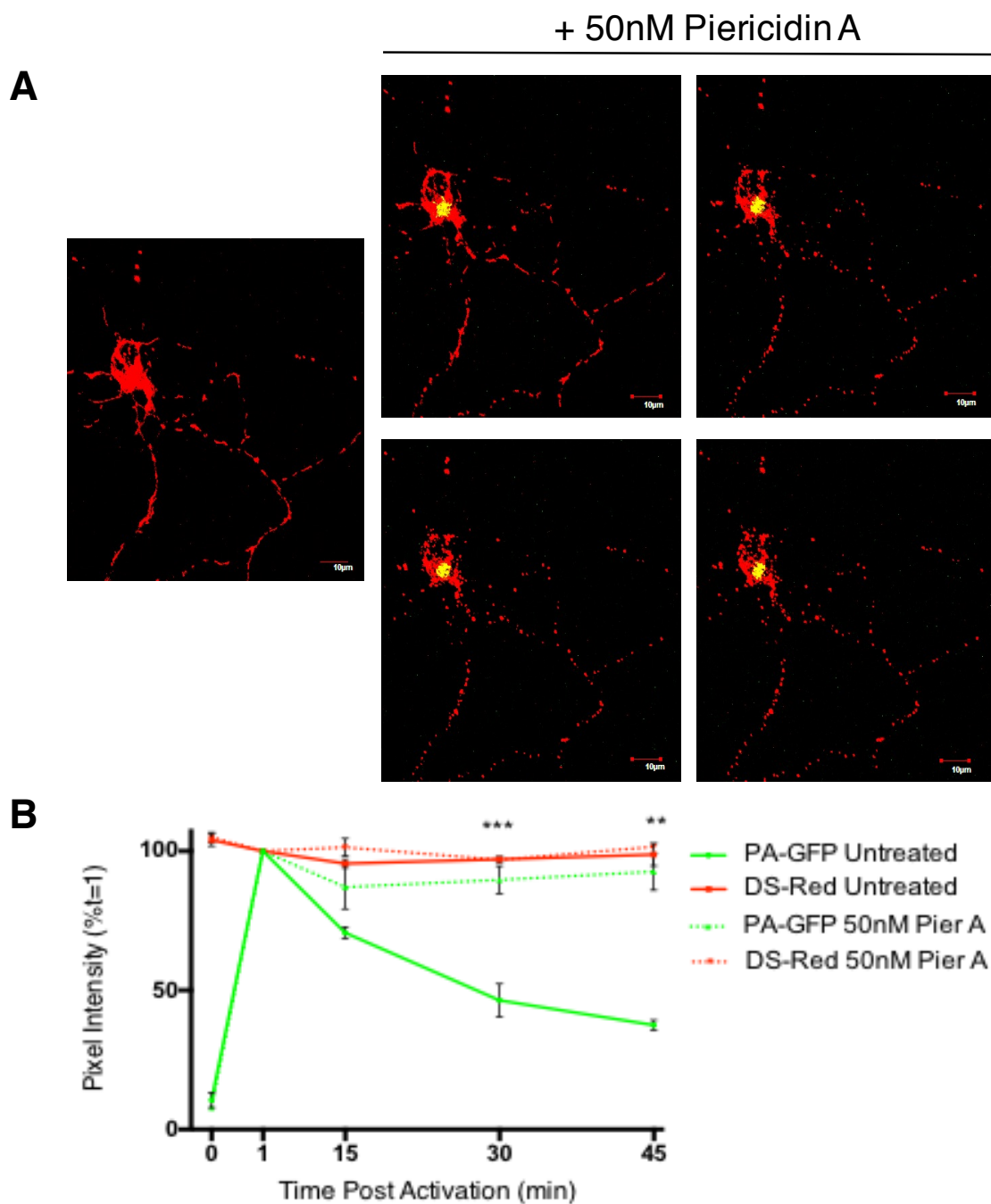


Figure 3.22: Inhibition of complex I with 50 nM piericidin A reduces mitochondrial fusion in primary cortical neurons. (A) Images representative of the effect of a 5 min pre-incubation with 50 nM piericidin A on mitochondrial fusion. (B) Quantitative data shows significant inhibition of fusion in piericidin A treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

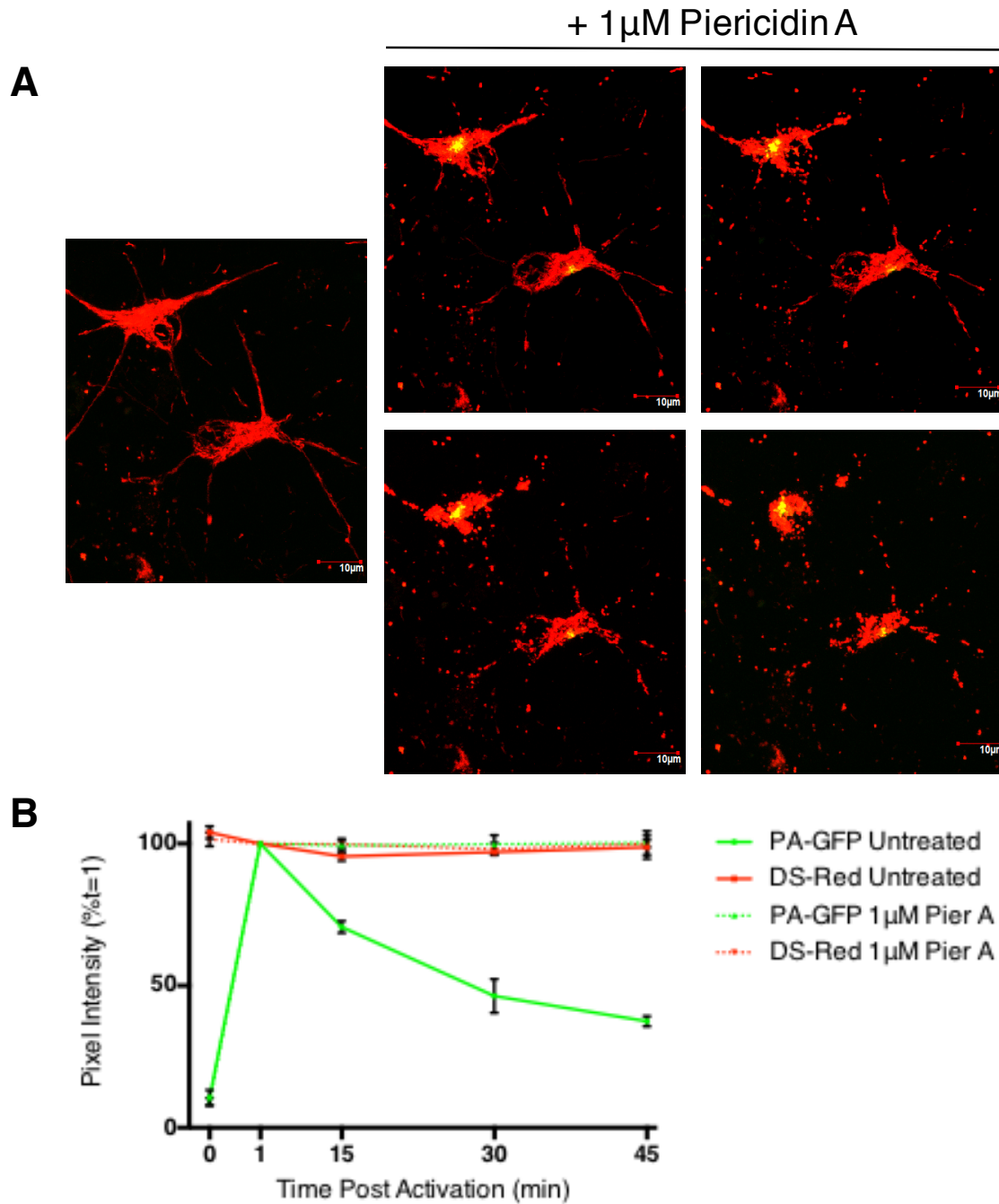


Figure 3.23: Inhibition of complex I with 1 μ M piericidin A reduces mitochondrial fusion in primary cortical neurons. (A) Images representative of the effect of a 5 min pre-incubation with 1 μ M piericidin A on mitochondrial fusion. (B) Quantitative data shows significant inhibition of fusion in piericidin A treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, n=3. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

3.3.7 Threshold Effect of Complex I Inhibition on $\Delta\psi_m$, ATP Production, ROS Production and Metabolism in Primary Cortical Neurons

Mitochondrial membrane potential experiments were carried out, as in PC12 cells, using microscopic measurement of TMRM fluorescence. As seen in Fig 3.22(A), there was a dose dependent decrease in $\Delta\psi_m$ after 5 min incubation with varying concentrations of piericidin A when compared to control. Statistically significant decreases were observed at all concentration above (and including) 20 nM, further highlight a link between decreases in $\Delta\psi_m$ and cessation of mitochondrial fission. Neuronal ATP levels were also assayed using a luminescent luciferase based assay. Results showed that neuronal ATP levels were significantly affected following 5 min exposure to piericidin A in a dose dependent manner. At a concentration of 100 nM, piericidin A caused a 32.82% decrease in cellular ATP, with a two-fold greater decrease at 500 nM and 1 μ M. Interestingly, there were no observed decreases in ATP content at 10 nM or 50 nM piericidin A. It is possible decreases were observed at later time points, but there were no immediate effects on ATP levels at this concentration.

Measurement of ROS production in primary cortical neurons identified no significant differences between control and piericidin A treated neurons at any concentration, as seen in Fig. 3.23, indicating this does not appear to be the primary cause for reduction in fusion in agreement with the literature (Baricault et al., 2007). OXPHOS was measured by a seahorse XF24 flux analyser which detects OCR of cell systems. Primary neurons were cultured in seahorse cell culture plates for 8 days and on the day of the experiment, medium was changed to seahorse medium and the plate was dehumidified while the cartridge plate was calibrated. Various concentrations of piericidin A were injected to the cell culture plate, after baseline OCR was measured, giving final concentrations of 10-50 nM. As can be seen from Fig. 3.24, OCR decreased after addition of piericidin A in a dose dependent manner. Due to fluctuating cell survival rates of primary neurons in seahorse plates, each well was normalized to a percentage of its baseline rates before the addition of the inhibitor. From these calculations, it can be seen that

10 nM piericidin A decreases oxygen consumption by 50.5% but it requires a decrease in oxygen consumption of 64.8% (at 20 nM piericidin A) before mitochondrial fusion is significantly compromised. This threshold seems high but it is also interesting to note that the ECAR is increased when CI is inhibited as the neurons try to compensate for reductions in oxygen consumption. Incubation with 20 nM piericidin A results in a 31.35% increase in ECAR suggesting a capability of neurons to shift their metabolism towards glycolysis if necessary. It should be noted that the results shown in the Fig. 3.24 are representative of an ~20 min incubation with piericidin A as a result of the standard protocol on the Seahorse machine which requires a period of mixing after injection of inhibitor. However, as there is little fluctuation between the 3 readings after addition of piericidin A, it can be concluded that the inhibitor acts quickly and maintains a steady level of inhibition.

Finally, to assess the degree of complex I inhibition at each concentration directly, complex I assays were carried out on NSM prepared from adult rat brain (Fig. 3.25 A). These figures are an approximation of the level of complex I inhibition in neurons as NSM contain mitochondria from glial cells as well as those from the soma of neurons. Incubation with 10 nM piericidin A decreased complex I activity by ~30% in NSM and the effect was dose dependent as 50 nM resulted in greater than 50% inhibition of complex I activity. It was later decided to carry out complex I activity assays on primary cortical neurons to give a more definitive degree of complex I inhibition and the results were striking as they showed a much lower threshold for disruption of mitochondrial fusion. In primary neurons, incubation with 20 nM piericidin A decreased complex I activity in excess of 50% which means that neurons can undergo a high level of complex I inhibition before mitochondrial fusion is compromised. When this CI inhibition was graphed against % fusion (where a PI decrease to ~39% 45 min post activation represents 100% fusion) it shows the threshold of complex I inhibition affecting mitochondrial fusion is between 52.02% and 54.5%. This threshold is significantly lower than those previously reported threshold effects of complex I inhibition on OXPHOS and respiration which was ~30% (Telford et al., 2009, Pathak and Davey, 2008)

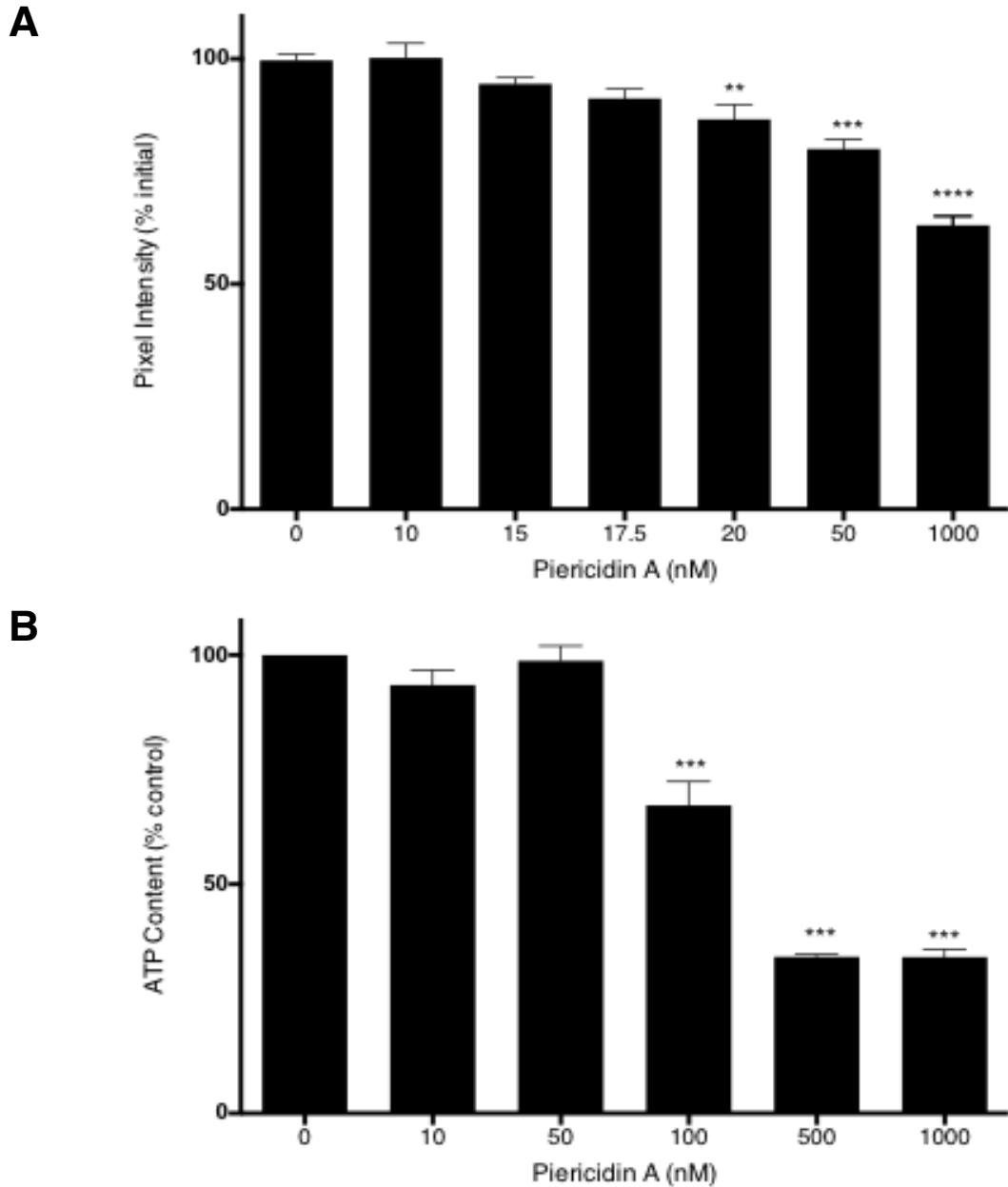


Figure 3.24: Inhibition of complex I with piericidin A causes partial mitochondrial depolarisation and decreases neuronal ATP in a dose dependent manner. (A) Neurons loaded with TMRM were exposed to piericidin A at the concentrations indicated for 5 min and exhibited significant reductions in $\Delta\psi_m$ from 20 nM onwards. (B) Neurons exposed to piericidin A for 5 min at the concentrations indicated showed a dose dependent decrease in ATP from 100 nM onwards (Data presented with permission from Stephen Quinn who carried out this experiment). Data presented as mean $\pm$ SEM, $n=3$ and expressed as % initial (pre-treatment) values for (A) and % control for (B). Ordinary one-way ANOVA followed by Dunnett's post comparison test for (A) and (B). Results which were significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

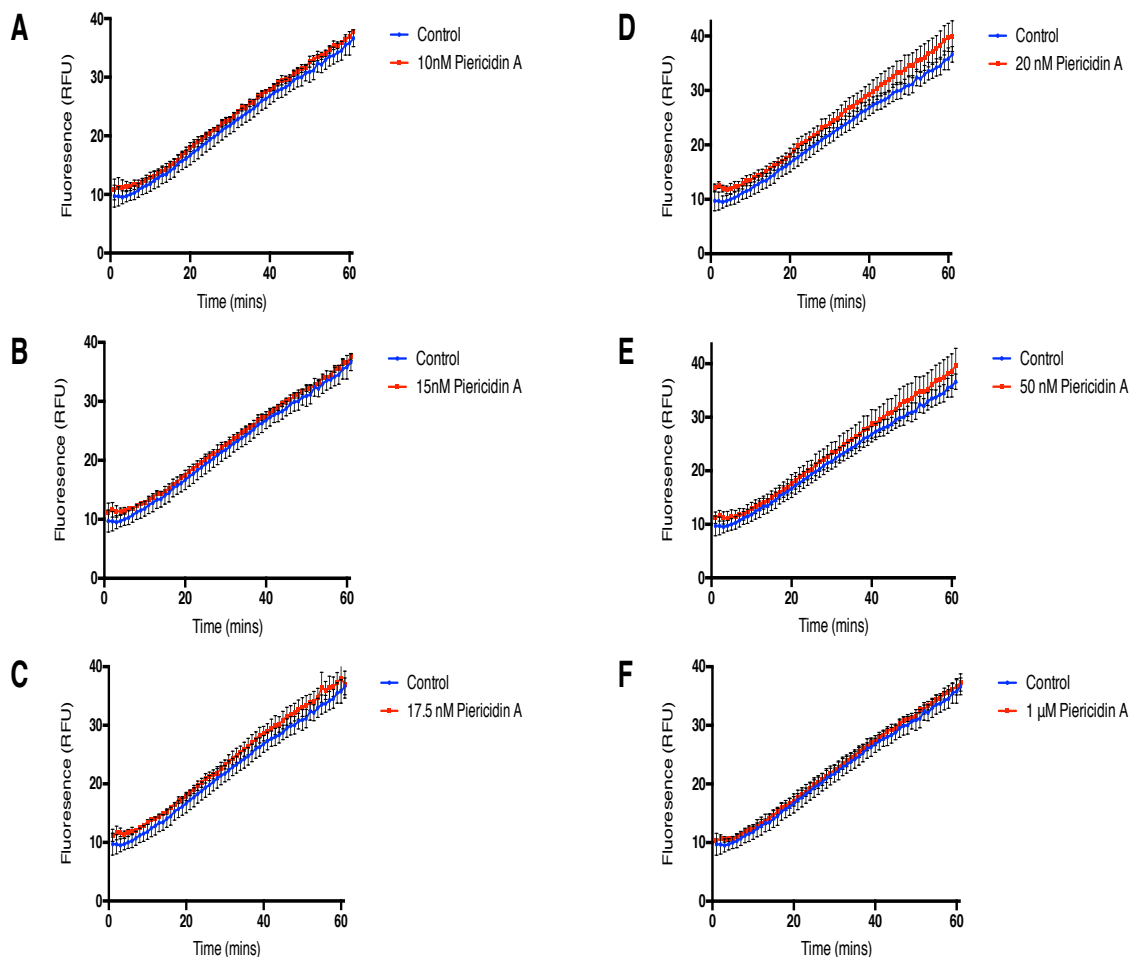


Figure 3.25: Inhibition of complex I with increasing concentrations of piericidin A has no effect on ROS production in primary cortical neurons. Cells were differentiated in clear-bottomed black 96 well plates for 8 days before being washed twice and incubated with $5\mu\text{M}$ H_2DCFDA in krebs buffer and assayed in a SpectraMax PLUS microplate spectrophotometer. Cells were incubated with H_2DCFDA in the presence of 10 nM piericidin A (A), 15 nM piericidin A (B), 17.5 nM piericidin A (C), 20 nM piericidin A (D), 50 nM piericidin A (E) or $1\mu\text{M}$ piericidin A (F) and compared to control wells loaded with battery. An individual t-test was performed at each time point and values that are significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

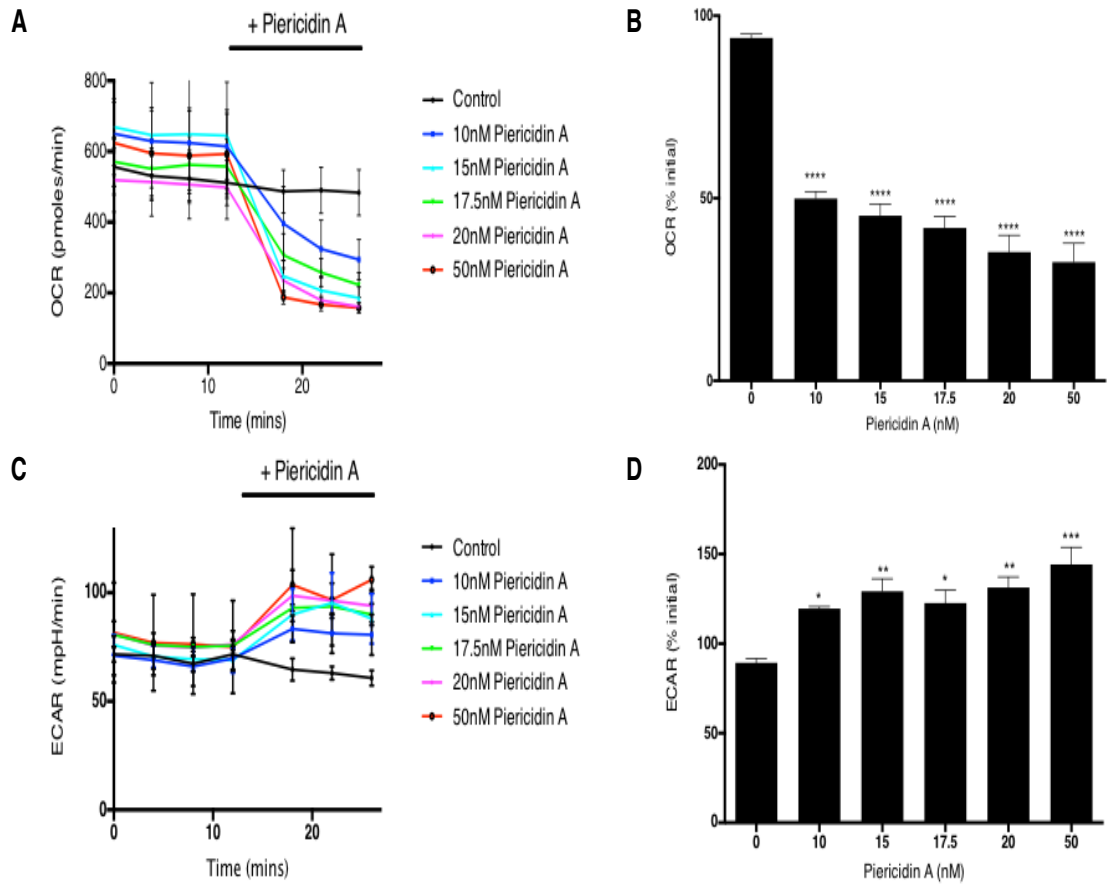


Figure 3.26: Inhibition of complex I with piericidin A decreases oxygen consumption rates and increases extracellular acidification rates in primary cortical neurons in a dose dependent manner. Cells were differentiated in an XF24 seahorse cell culture plate coated with poly-d-lysine for 8 days in neurobasal medium A before being switched to seahorse medium 45 min prior to the start of the experiment. Seahorse cartridges were loaded with various concentrations of piericidin A to give final concentrations as displayed. (A) Shows oxygen consumption rate (OCR) in pmoles/min before and after the injection of piericidin A. (B) Represents the OCR expressed as a percentage of initial values as a result of varying levels of cell survival from well to well. (C) Shows extracellular acidification rate (ECAR) as a measure of glycolysis before and after the addition of piericidin A. (D) Represents the ECAR expressed as a percentage of initial values as a result of varying levels of cell survival from well to well. All graphs show a clear dose dependent decrease in OCR after piericidin A injection which is accompanied by an increase in ECAR rates. Data presented as mean $\pm$ SEM, $n=3$. Ordinary one-way ANOVA followed by Dunnett's post comparison test for (B) and (D). Results which were significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

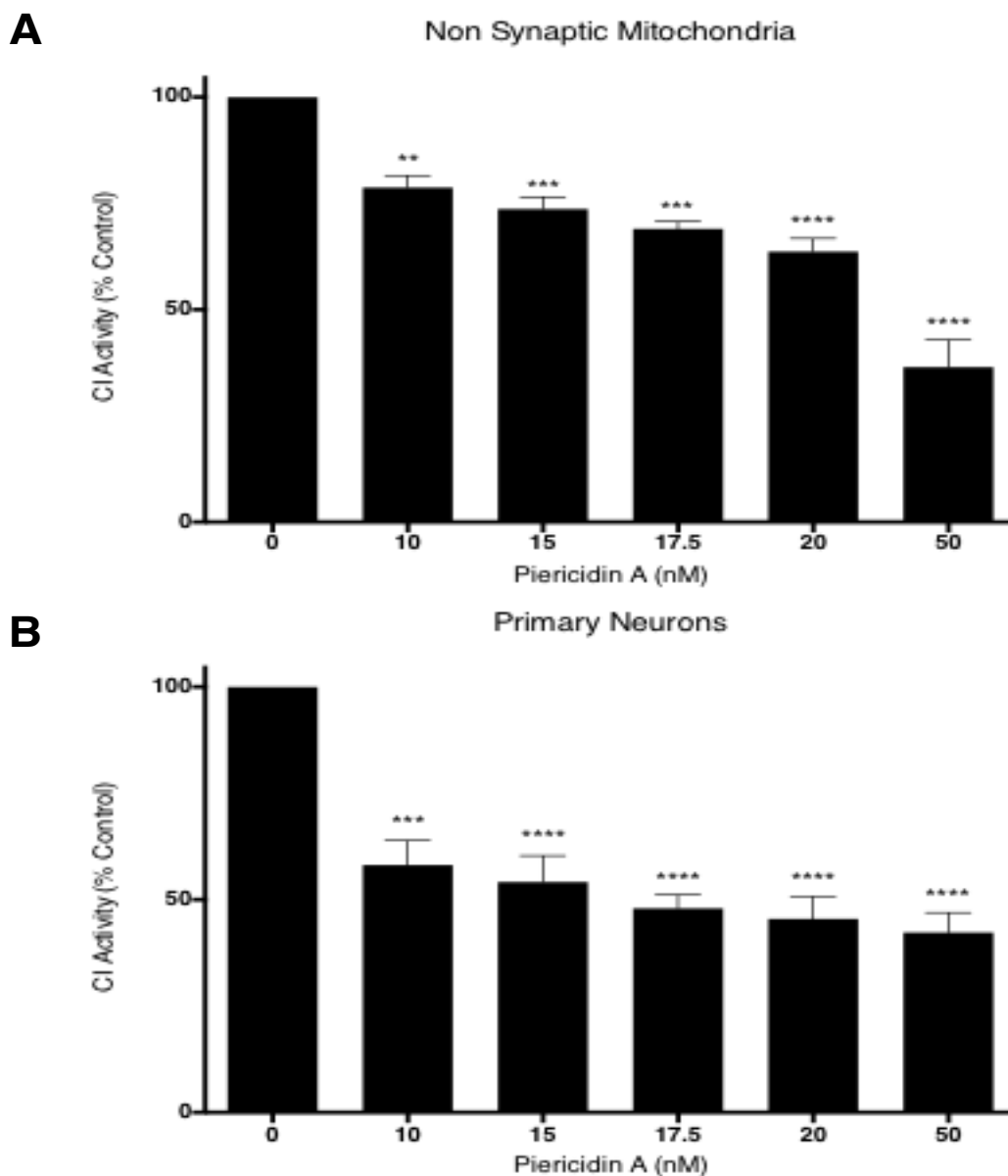


Figure 3.27: Piericidin A decreases complex I activity in a dose dependent manner in non-synaptic mitochondria and primary cortical neurons. Complex I activity was assayed spectrophotometrically in NSM and cortical neuron homogenate where a decrease in absorbance ($\Delta A_{340}/\text{min}$) indicates a rapid oxidation of NADH by complex I after the addition of an artificial electron acceptor, decylubiquinone. Samples were assayed in the absence or presence of piericidin A at the concentrations shown. After following the reaction for 3 min, the rotenone insensitive rates were obtained by monitoring the $\Delta A_{340}/\text{min}$ after the addition of 10 μM rotenone. Complex I activity, presented as % control (untreated), in NSM (A) and primary cortical neuron homogenate (B) at the piericidin A concentrations indicated. Data presented as mean $\pm$ SEM, $n=3$. Ordinary one-way ANOVA followed by Dunnett's post comparison test. Results which were significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

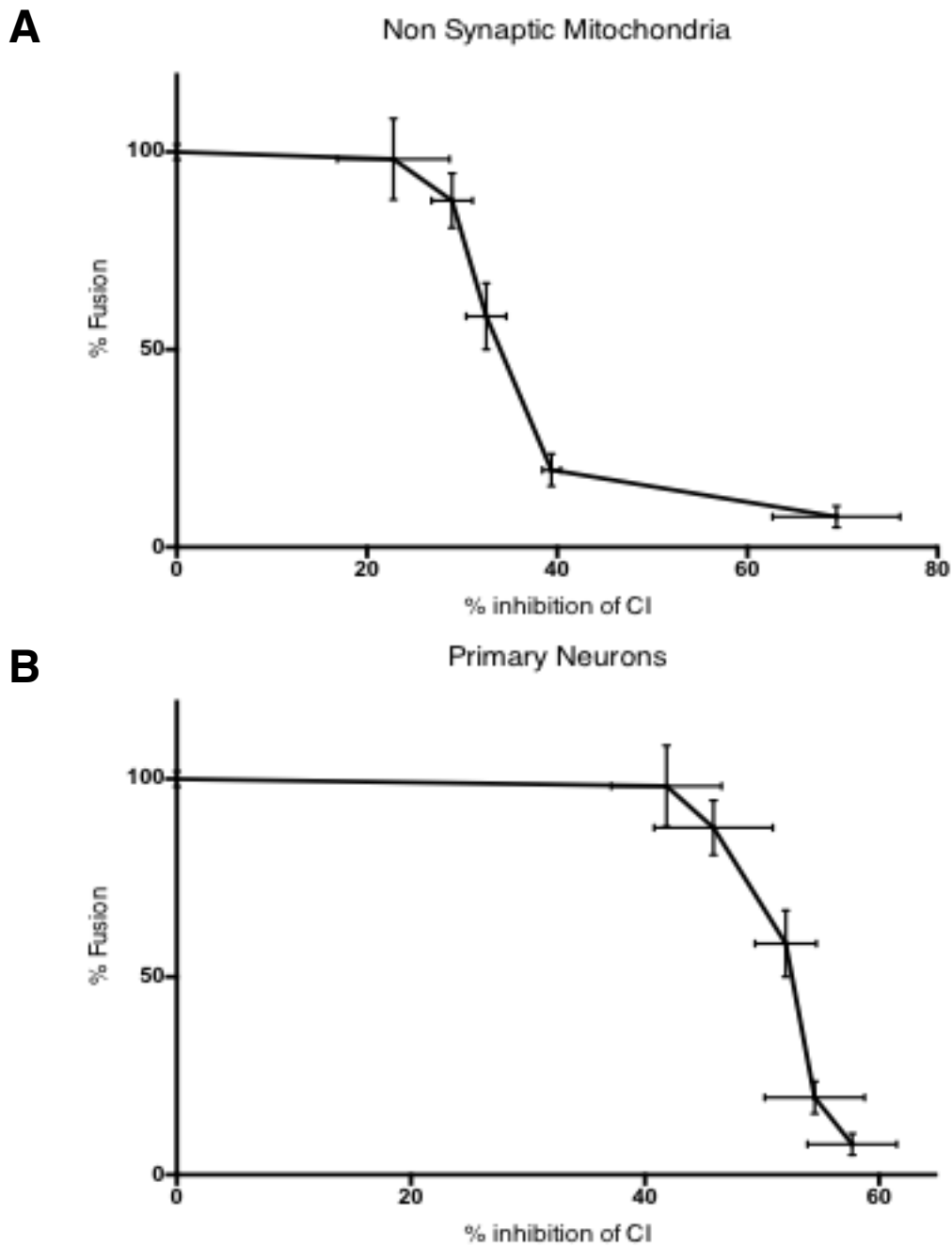


Figure 3.28: Complex I inhibition threshold and mitochondrial fusion. When % complex I inhibition [(A) NSM and (B) primary cortical neurons] is plotted against fusion (where a pixel intensity decreased to ~39% post-activation value represents 100% fusion), it appears that complex I has an inhibition threshold of between 32.54-39.4% in NSM, and 52.02% and 54.5% in primary cortical neurons before mitochondrial fusion is significantly compromised. Data presented as mean $\pm$ SEM, n=3

3.3.7 Threshold Effect of Complex I Inhibition on Mitodynamin Expression in Primary Cortical Neurons

As a result of the processing of Opa1 seen in differentiated PC12 cells after OXPHOS inhibition, it was decided to investigate if the same post translational modifications were occurring in primary neurons with non-fusing mitochondria. Five min treatment of neurons with the same concentrations of piericidin and subsequent lysis of cells before being analysed by western blot resulted in a clear increase in processing of Opa1. In the neurons treated with 20 nM and 50 nM piericidin A the ratio of L-Opa1: S-Opa1 is decreased and there is an obvious increase in S-Opa1 as identified via western blot. Interesting, treatment with 17.5 nM piericidin A results in a non-significant decrease in the ratio which may explain the subtle decrease in fusion seen with this level of treatment, highlighting that this level of CI inhibition is right on the threshold of what the cell can endure before fusion is seriously compromised.

There were subtle insignificant changes in expression levels of Drp1 and Mfn1/2 but as discussed above, this is not surprising considering these proteins are from whole cell lysates as opposed to mitochondrial fractions. Interestingly, when Drp1 was visualised using immunocytochemistry there was an accumulation of the protein around the mitochondria of the cell soma suggesting it was being recruited to the mitochondria in non-fusing neurons as shown in Fig. 3.30. In untreated neurons, and those treated with 10 nM piericidin A, Drp1 is distributed throughout the cell in an even manner. However, when treated with 50 nM piericidin A, 2 μ M antimycin A or 5 μ M FCCP, there is a clear co-localization with mitochondria around the nucleus which goes some way to explaining the fragmentation of the mitochondrial network seen during the fusion assays.

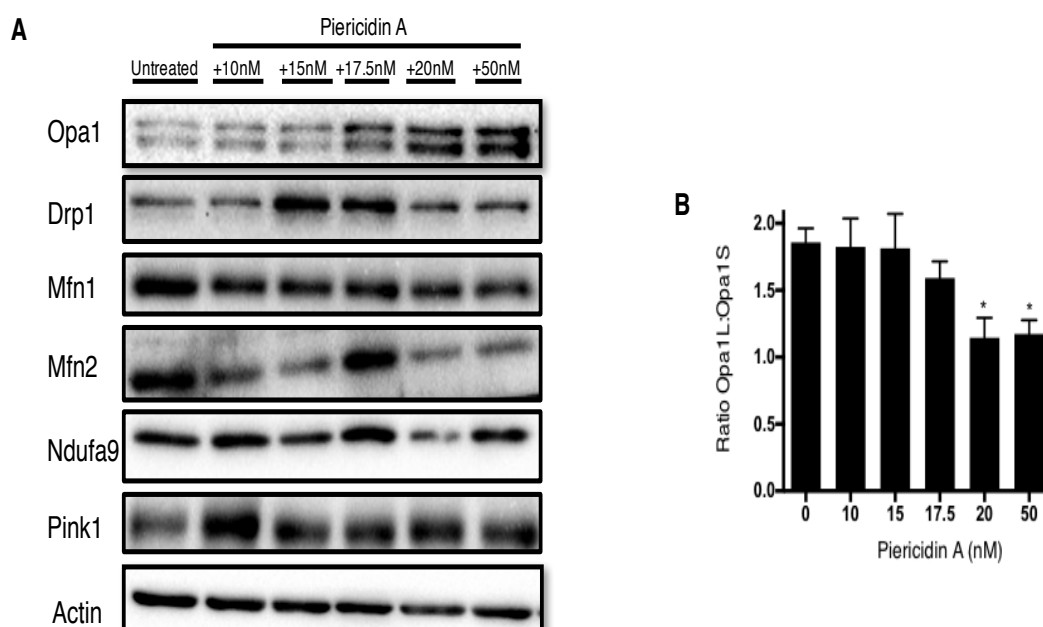


Figure 3.29: Expression of the fusion/fission machinery remains unchanged except for alterations in Opa1 isoforms at higher concentrations of piericidin A in primary cortical neurons. (A) Neurons cells were differentiated for 8 days and treated with vehicle (untreat), 10 nM, 15 nM, 17.5 nM, 20 nM and 50 nM piericidin A for 5 min before being lysed with RIPA buffer and analysed for fusion mediating mitodynaminis by western blot. No significant changes were observed in protein levels of Drp1, Mfn1 or Mfn2 but changes in the long and short forms of Opa1 were observed. Images representative of n=3. (B) Densitometry analysis of Opa1 western blots shows significant changes in the ratio of the long form: short form. In control samples, there is twice as much of the long form as the short form but in cells treated with 20 and 50 nM piericidin A the ratio decreases to around 1. Ordinary one-way ANOVA followed by Dunnett's post comparison test. Data presented as mean $\pm$ SEM, n=3. Results which were significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

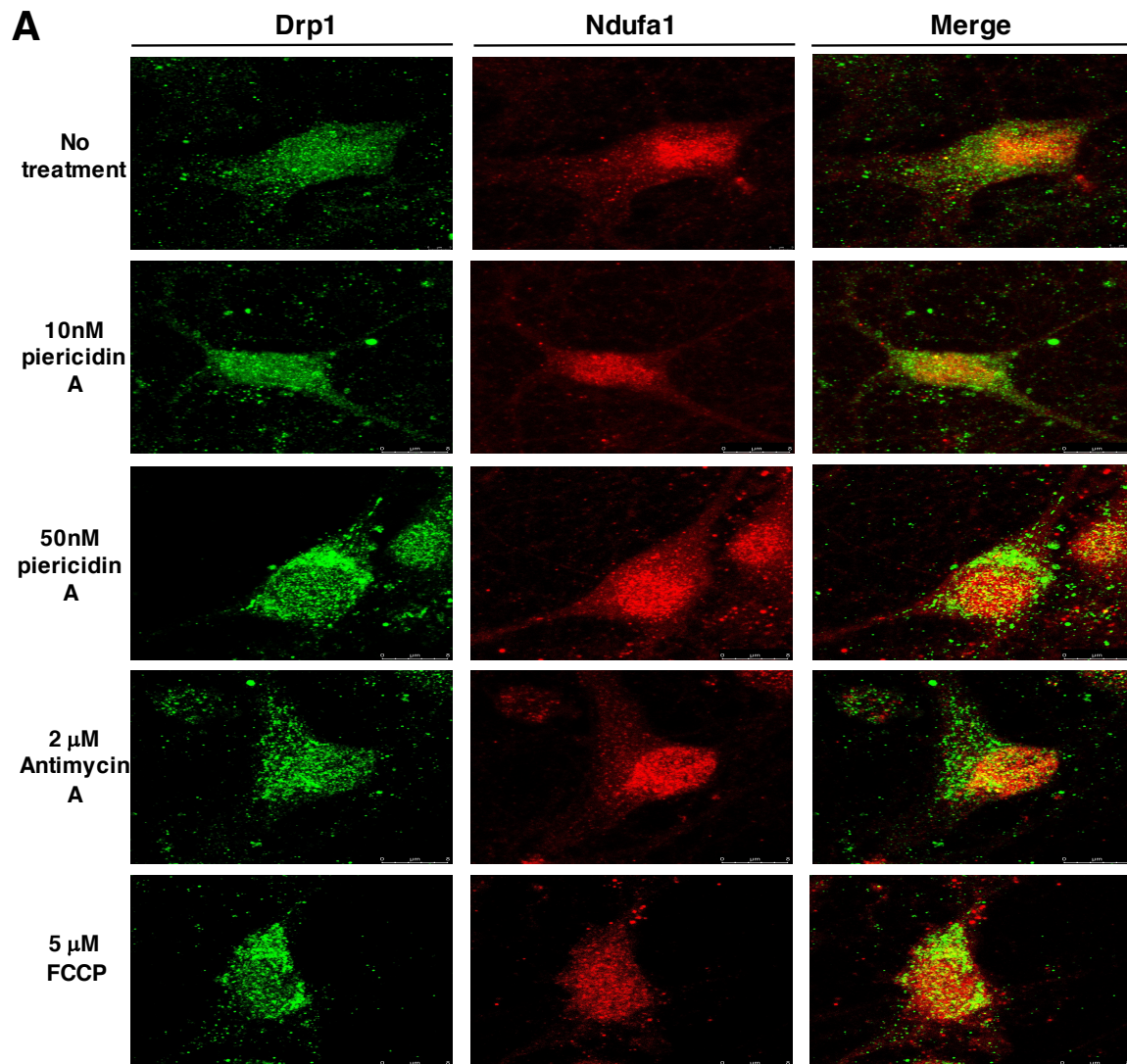


Figure 3.30: Drp1 localizes to mitochondria in the perinuclear area in non-fusing mitochondria in primary cortical neurons. Primary cortical neurons were cultured for 8 days on poly-d-lysine coated glass coverslips before being treated with 10 and 50 nM piericidin A, 2 μ M antimycin A or 5 μ M FCCP for 5 min, washed with PBS, fixed with 4% PFA and permeabilized with 0.1% triton X-100. Cells were then blocked for 1 hour and incubated with primary antibodies against Drp1 and Ndufa9 (complex I subunit) overnight and secondary antibody for 1 hour the following day before being mounted on slides for imaging. In untreated cells and those treated with 10 nM piericidin A, Drp1 is distributed throughout the cell and does not appear to accumulate too closely with mitochondria. However, in those cells treated with 50 nM piericidin A, 2 μ M antimycin A or 5 μ M FCCP Drp1 appears to localize to mitochondria in the perinuclear area. Images representative of n=3.

3.3.8 Ndi1 Rescues Inhibition of Mitochondrial Fusion Caused by 50 nM piericidin A in Primary Cortical Neurons.

NADH-quinone oxidoreductase (Ndi1) is a nuclear gene from *Saccharomyces cerevisiae* which encodes a single subunit complex I equivalent which is imported into mitochondria (Kitajima-Ihara and Yagi, 1998). Ndi1 is rotenone (and piericidin A) insensitive and so can maintain the PMF even in the presence of common complex I inhibitors. Primary cortical neurons were transfected with Ndi1 and empty vectors controls and a mitochondrial fusion assay was carried out. With the empty vector, it was found that incubation with 50 nM piericidin A was sufficient to abolish mitochondrial fusion. However, as can be seen in fig. 3.31, in cells transfected with humanized Ndi1, treatment with 50 nM had no significant effect on fusion when compared to control cells. 45 min after photo-activation, PI levels had reduced to 54.01% of the intensity seen 1 min post activation compared to 40.2% seen in controls. This represents a restoration of fusion to 76% of control levels as opposed to the 14% seen in neurons transfected with empty vector and treated with 50 nM piericidin A. It should also be noted that neurons have a low transfection rate, so Ndi1 may fully restore fusion rates but it cannot be determined if every cell on which the assay is carried out has been successfully transfected.

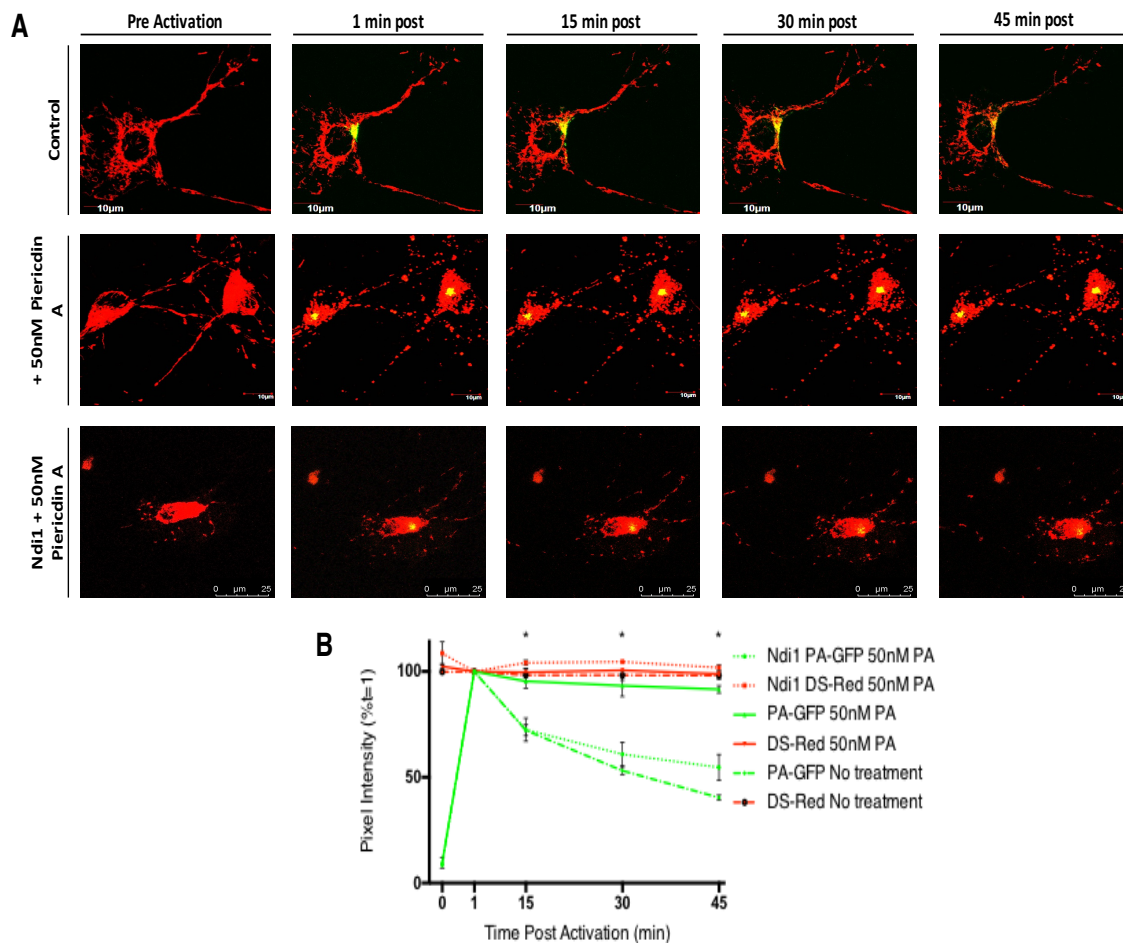


Figure 3.31: Ndi1 rescues complex I inhibition of mitochondrial fusion in primary cortical neurons. (A) Images representative of the effect of control fusion rates (top panel), neurons treated with 50 nM piericidin A (middle panel) and neurons transfected with Ndi1 and treated with 50 nM piericidin A. (B) Quantitative data shows significant rescue of inhibition of fusion in Ndi1 transfected piericidin A treated cells (dashed green line) compared to controls treated with piericidin A (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

3.3.9 Compounds and Mutations Associated with Parkinson's Disease Reduce Mitochondrial Fusion in Differentiated PC12 cells

Complex I dysfunction was first associated with PD when individuals who consumed a toxic by-product called MPTP developed parkinsonian symptoms soon after. MPTP is oxidised by monoamine oxidase in glial cells to MPP⁺ (Singer and Ramsay, 1990). Here we show that direction incubation of differentiated PC12 with MPP⁺ cells decreases mitochondrial fusion by ~55% (Fig 3.32). Curiously, the inhibition was not as great as seen as incubation with rotenone or piericidin A (Fig 3.5 and 3.6) despite a well characterized inhibition of complex I by this compound. This is likely because MPP⁺ slowly penetrates the hydrophobic reaction site on NADH dehydrogenase and so a longer incubation time would decrease the fusion rate further. PC12 cells over-expressing wild type and mutant (A53T) α -synuclein were generated in combination with another student in the lab (Dr. Natalie Adlesic). Mutations in α -synuclein have been associated with early onset PD for many years yet little is known about the toxic mechanism of the protein (Polymeropoulos et al., 1997). Overexpression of wild type α -synuclein results in a significant decrease in mitochondrial fusion in differentiated PC12 cells (Fig 3.33). Similarly, as shown in Fig 3.34, overexpression of mutant (A53T) α -synuclein results in a significant reduction in mitochondrial fusion as shown by the difference in pixel intensity 45 min post activation in differentiated PC12 cells. These results demonstrate that agents known to be causative of PD, decrease mitochondrial fusion, likely through inhibition of complex I.

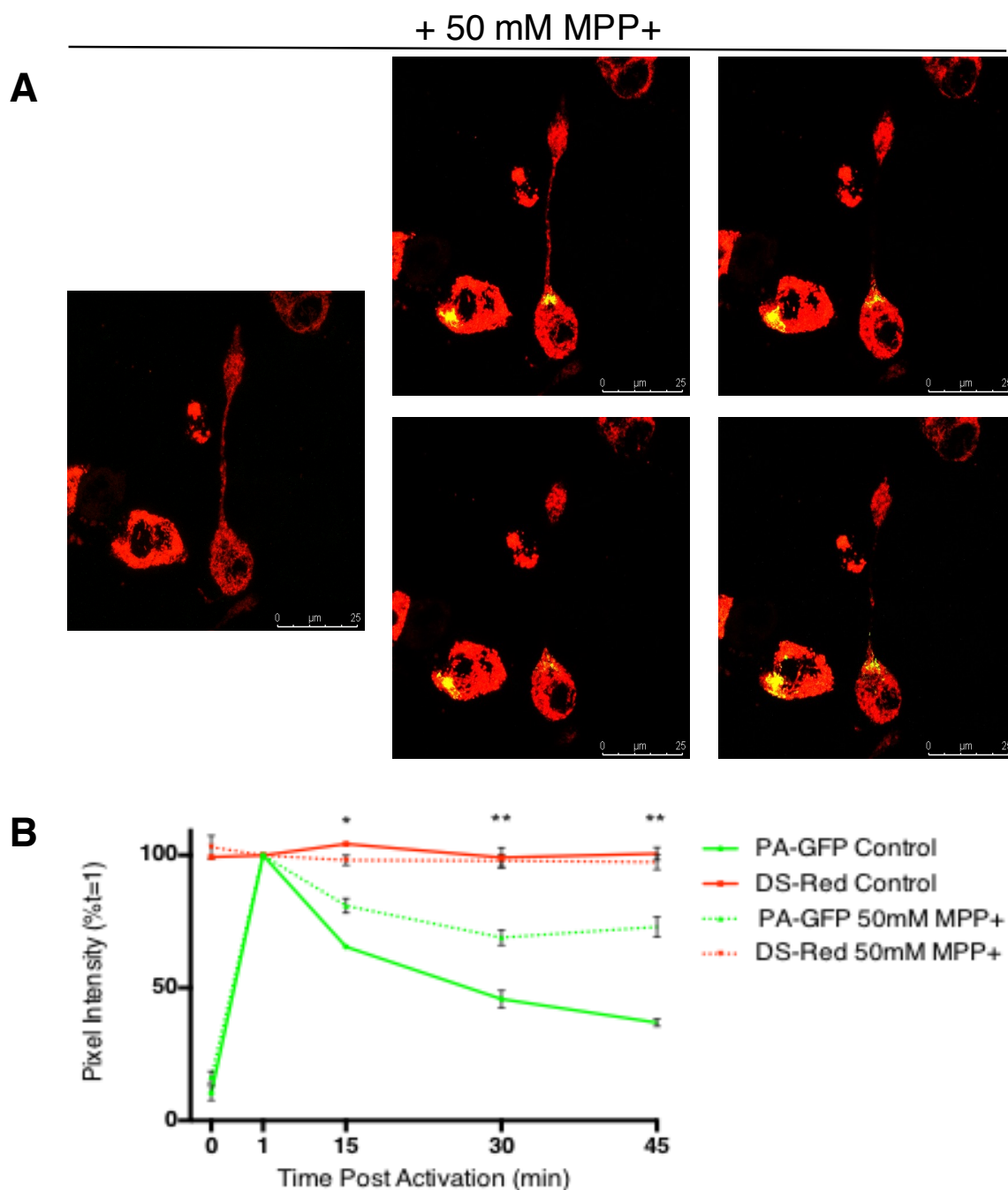
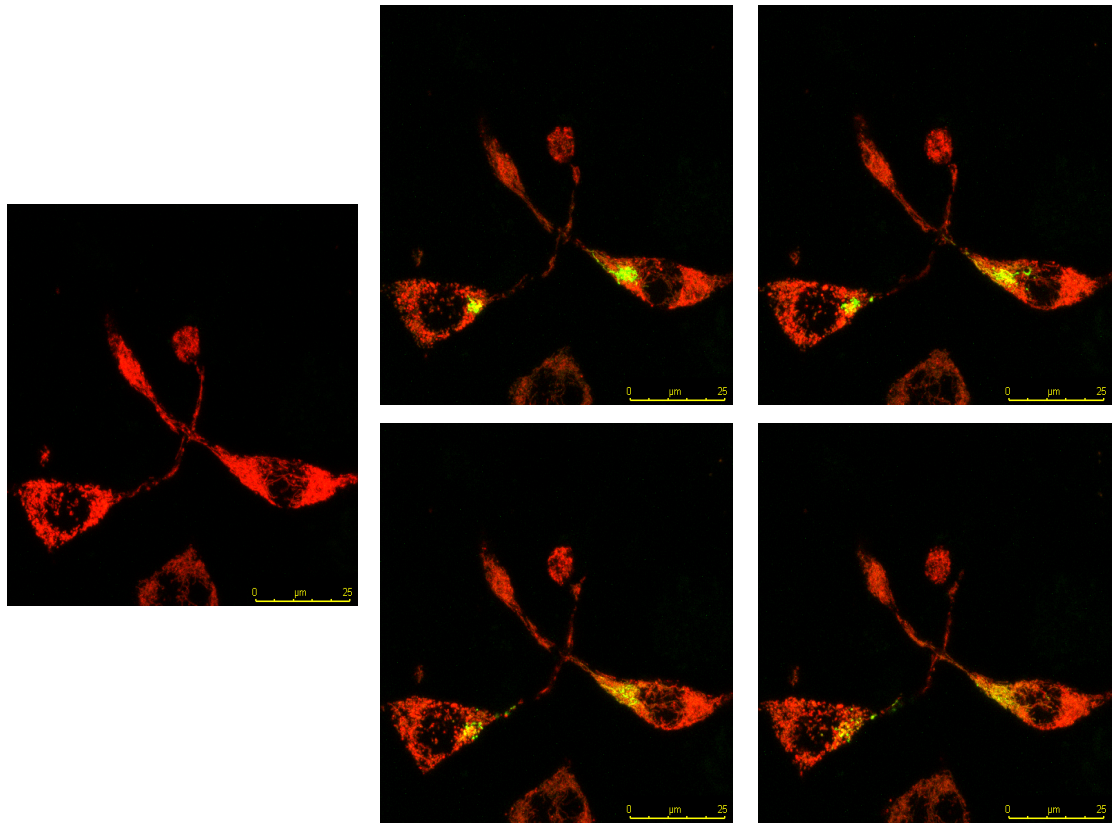


Figure 3.32: Inhibition of complex I with 1-methyl-4-phenylpyridinium reduces mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 20 min pre-incubation with 50 mM MPP⁺ on mitochondrial fusion. (B) Quantitative data shows inhibition of fusion in MPP⁺ treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, n=3. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

A



B

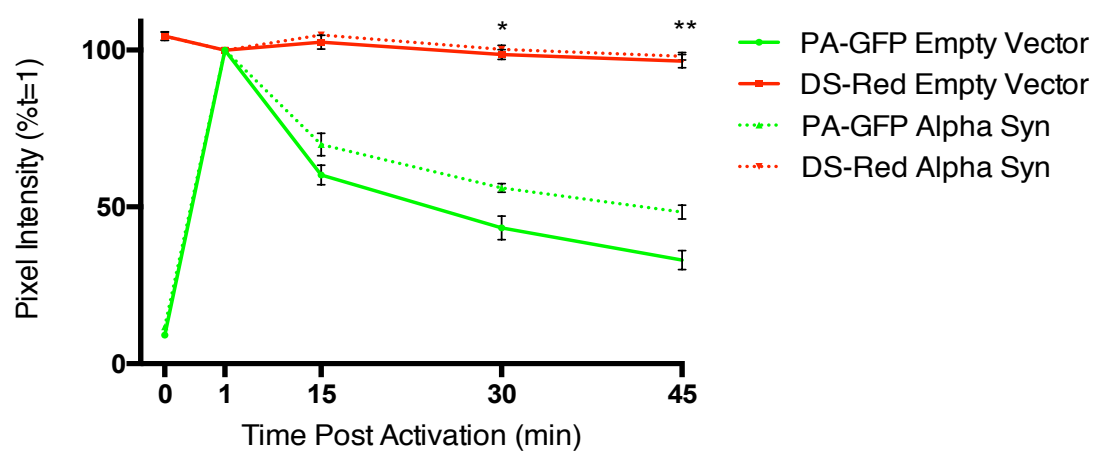


Figure 3.33: Overexpression of alpha-synuclein reduces mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of stable overexpression of alpha-synuclein on mitochondrial fusion. (B) Quantitative data shows inhibition of fusion in cells overexpressing wild-type alpha synuclein (dashed green line) compared to empty-vector controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

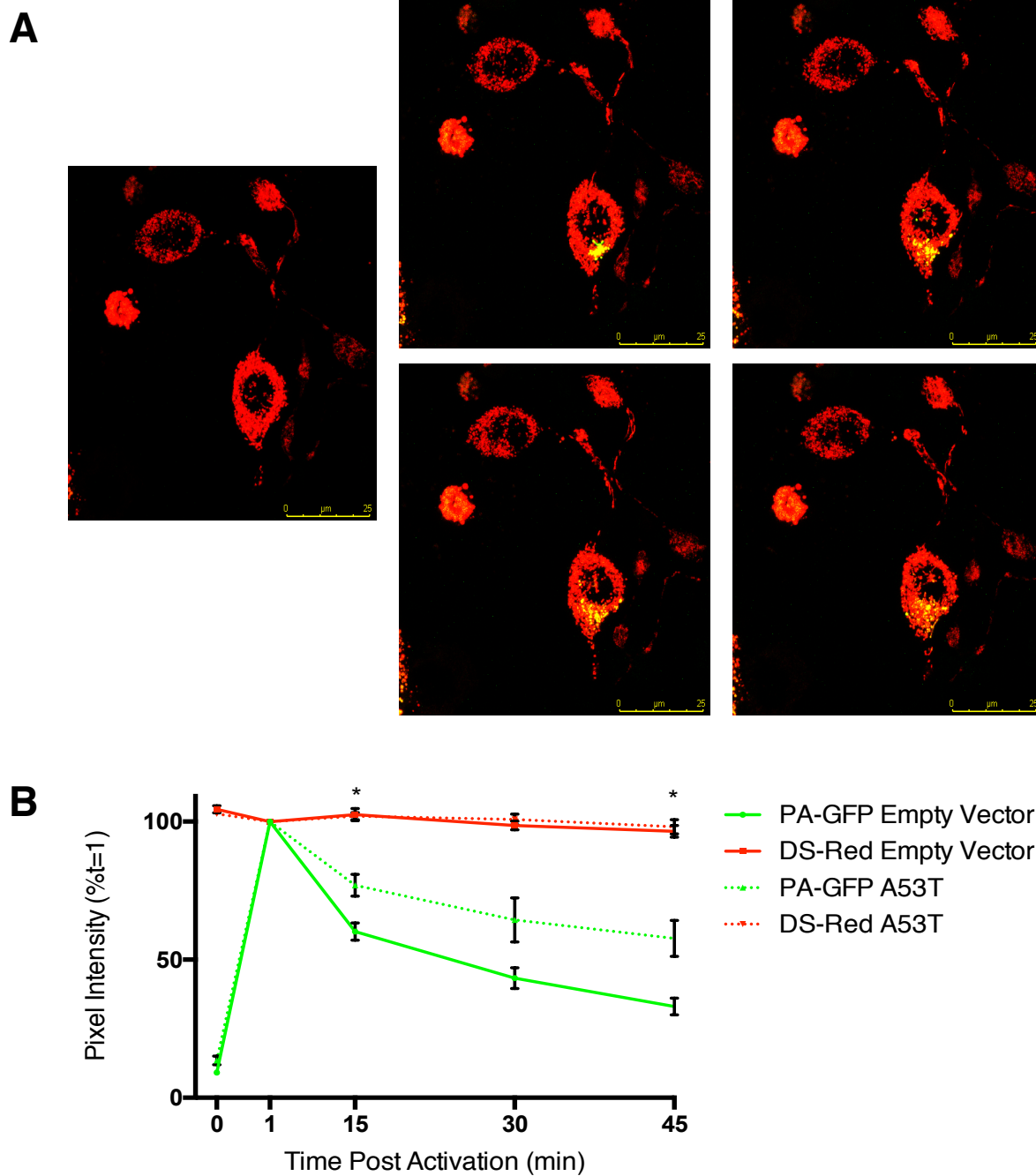


Figure 3.34: Overexpression of mutant (A53T) alpha-synuclein reduces mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of stable overexpression of mutant (A53T) alpha-synuclein on mitochondrial fusion. (B) Quantitative data shows inhibition of fusion in cells overexpressing mutant (A53T) alpha synuclein (dashed green line) compared to empty-vector controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

3.4 Discussion

Mitochondrial fusion is a vital process in neurons, which protects mitochondrial function and allows for spatial distribution of mitochondria, especially in areas of high energy requirement, such as the synapse and dendritic spines (Chen and Chan, 2010). In recent years, the interplay between bioenergetics and mitochondrial dynamics has begun to be investigated but as yet, no study has shown that inhibition of the ETC blocks mitochondrial fusion despite numerous studies showing that ETC inhibition results in a fragmentation of the mitochondrial network in a variety of cell types (Barsoum et al., 2006, Kaasik et al., 2007, Safiulina et al., 2006). This study highlights the extreme vulnerability of neurons to bioenergetic inhibition. Mitochondrial fusion was completely blocked by OXPHOS inhibition, which was concomitant with a rapid dissipation of $\Delta\psi_m$. In addition, it was shown that 5-minute inhibition of complex I had dose dependent effects on fusion, $\Delta\psi_m$ and ATP levels and, when plotted against complex I inhibition in primary cortical neurons, neurons could withstand ~53% inhibition of complex I before fusion was perturbed.

The control fusion rates observed in differentiated PC12 cells and primary cortical neurons are slower than those reported in other cell types but faster than expected in neurons. Berman et al. (2009) estimated that only 1-2% of encounters between mitochondria in mouse primary cortical neurons resulted in fusion events while Karbowski et al. (2004) reported that primary neurons require an average of 1 hour to decrease PIs to the same level achieved in HeLA cells in 30 minutes. The most likely reason for this is that the above studies measured fusion rates in the axons of neuronal cells where mitochondrial contact is less likely to occur and most mitochondria are anchored to the microtubule network moving in an anterograde or retrograde direction for a specific purpose and so would be less likely to fuse until they reach their destination; ie. an area with higher energy demands (Schwarz, 2013). It should also be noted that this technique is semi-quantitative and can be difficult to compare across labs where laser strengths and acquisition settings are likely to be slightly different.

However, in general, the results presented above agree with other publications that have found post mitotic cells have lower mitochondrial fusion rates than proliferating cells such as HeLa and human umbilical vein endothelial cells (HUVECs) (Jendrach et al., 2005) (Davey, unpublished data). The reason for this phenomenon is not known although Chang and Reynolds (2006) found that neuronal mitochondria may be more dynamic during growth and development to ensure extensive branching and interconnectivity while the maintenance of mature neurons requires less active turnover. The same paper also found that younger neurons expressed higher levels of Mfn1 and lower levels of Drp1 than their older counterparts while Eura et al. (2003) found that Mfn2 is expressed in preference of Mfn1 in rat brain. Interestingly, it has also been displayed that homotypic Mfn complexes are less likely to fuse than heterotypic ones (Hoppins et al., 2011), so it stands that younger neuronal cells express higher levels of Mfn1 to promote the formation of heterotypic Mfn complexes and encourage fusion during growth and development while a gradual decrease in Mfn1/increase in Mfn2 expression in older neurons is responsible for the slower fusion rates observed.

Exposure of differentiated PC12 cells to OXPHOS inhibitors had obvious effects on both mitochondrial morphology and mitochondrial fusion (Fig 3.4-3.10). Inhibition of Complexes I, III and IV, as well as ATP synthase, resulted in almost a complete abolition of mitochondrial fusion. Although there is no evidence to suggest that any subunits of any of the components of the ETC are necessary for fusion, a high energy process that generates a constant supply of ATP and GTP is thought to be required (Hoppins and Nunnari, 2009). It could be argued that the decrease in fusion rates observed could be as a result of a decrease in mitochondrial motility, owing to a lack of energy supply (both kinesin and dynein are ATPases (Wang and Sheetz, 2000)), which would decrease the number of contact events between mitochondria. This would tip the balance in favour of fission and one would expect to see more punctate mitochondria as fissions events continue as is seen when complexes I, III and ATP synthase are inhibited

and the $\Delta\Psi_m$ is collapsed in this study. Many other studies of mitochondrial morphology have shown that inhibition of the ETC with rotenone (Barsoum et al., 2006), oligomycin (Kaasik et al., 2007), antimycin A, azide and FCCP (Safiulina et al., 2006) results in fragmentation of neuronal mitochondria. These effects are seen here, although they are not as pronounced as seen in other cell types and inhibition of CIV failed to elicit fragmentation, indicating that this effect may occur at a slower rate in differentiated PC12 cells than the neuronal cells described in the literature.

According to the literature, the metalloprotease Opa1 is the primary link between bioenergetics and mitochondrial dynamics and the results obtained here support this hypothesis. Opa1 is a dynamin-like GTPase controlling inner membrane fusion, the long form of which is proteolytically cleaved under normal conditions, to produce a balanced accumulation of L-Opa1 and S-Opa1 isoforms (Ishihara et al., 2006). However, under stress conditions, L-Opa1 is completely cleaved into S-Opa1 which inhibits fusion, the best characterised example of this is through the addition of an ionophore such as FCCP to dissipate $\Delta\Psi_m$ and prevent fusion. Here, we have shown that 20 min incubation with a variety of OXPHOS inhibitors is sufficient to induce a significant change in the ratio of L-Opa1 to S-Opa1 and abolish mitochondrial fusion in differentiated PC12 cells and in primary neurons. Interestingly this incubation time was not sufficient to completely cleave L-Opa1, yet did result in an almost complete cessation of mitochondrial fusion, suggesting that an accumulation of S-Opa1 may be sufficient to inhibit fusion in neuronal models. Oma1 is the main metalloprotease responsible for Opa1 cleavage under stress conditions and Baker et al. (2014) propose that Oma1 accumulates on the MOM and a sequence of positively charged amino acids on the N-terminal acts a sensor domain, detecting membrane depolarization and subsequently inducing a conformational change leading to its activation and import into the MIM where it can rapidly cleave L-Opa1. Based on these results, it would be expected that a longer incubation with OXPHOS inhibitors would result in a more complete processing of Opa1 but that a moderate increase in S-Opa1 can inhibit fusion. Others have shown that inhibition of OXPHOS with antimycin A or rotenone can

induce a decrease in $\Delta\psi_m$ which is not accompanied by a cleavage of L-Opa1 (Baker et al., 2014, Guillery et al., 2008). Our results show that neuronal cells are more susceptible to Opa-1 processing through a loss of $\Delta\psi_m$ and that this is sufficient to completely abolish mitochondrial fusion, as demonstrated through our fusion assay. Although oligomycin does not decrease $\Delta\psi_m$, recent studies have shown that Opa-1 cleavage can be activated by a variety of cellular stress responses, including lowered ATP levels, and that these metabolic stresses can activate Oma1 (Baricault et al., 2007). Indeed, Baker et al. (2014) showed that inhibition of ATP synthase with oligomycin in MEFs resulted in a partial cleavage of OPA1, which was Oma1 dependent despite no significant change in $\Delta\psi_m$. In spite of this, it should be considered that all OXPHOS inhibitors used would likely result in a decrease in ATP levels. The dynein and kinesin motors used to facilitate mitochondrial movement are ATPases and so it is likely that some of the reduction in mitochondrial fusion seen is through a reduction or complete halt of mitochondrial motility, thus reducing the likelihood of a fusion event occurring. Activation of Oma1 is accompanied by its autocatalytic degradation, so it would be interesting to perform some time dependent wash-out experiments with reversible ETC inhibitors to see how quickly the ratio of L-Opa1:S-Opa1 could return to control levels and how quickly mitochondrial network could be repaired. It would also be useful to run cell lysates for longer, on a lower percentage acrylamide gel, to identify different isoforms of S-Opa1 which may be responsible for the cessation of mitochondrial fusion seen in differentiated PC12 cells and neurons.

The cessation of fusion is unlikely to be the sole reason for the fragmentation of the mitochondrial network seen after inhibition of complex I, III and ATP synthase in differentiated PC12 cells and CI in neurons (additional data from the lab shows fragmentation occurring upon inhibition of CIII, CIV and ATP synthase in neurons) as activation of Drp1 is also likely to have occurred. Rotenone (Swarnkar et al., 2012) and antimycin A (Plaisance et al., 2003) have been shown to increase cytosolic Ca^{2+} in neuronal cells which could promote calcineurin-dependent dephosphorylation of Drp1 at serine 637, thus enhancing its fission activity

(Cribbs and Strack, 2007). In this study, we do not see any significant changes in Drp1 protein expression in whole cell lysates after a 20 min incubation with OXPHOS inhibitors (Fig. 3.17). It would be interesting to look at Drp1 protein expression in a mitochondrial fraction from these cells as we can see in Fig. 3.30 that Drp1 appears to localize with mitochondria after short incubation with piericidin A, antimycin A and oligomycin in primary neurons, agreeing with the literature that recruitment of Drp1 to the mitochondria is the trigger for fission as opposed to *de novo* protein synthesis (Otera et al., 2010). It would be useful to use a different Drp1 antibody to identify any increases in phosphorylation at S616 or decreases in phosphorylation at S637 as a trigger for increased Drp1 activity. There is little change in Mfn1/2 protein expression in either PC12 cells or neurons which is in agreement with the literature suggesting their activity is regulated by other cellular responses and not metabolic activity.

Our results show the existence of a threshold of inhibition of complex I on mitochondrial fusion in primary cortical neurons. Pre-incubation with 20 nM piericidin A for 5 min prior to photo-activation of a ROI abolishes mitochondrial fusion but pre-incubation with 17.5 nM piericidin A has no significant effect on fusion rates. Treatment with 10 nM and 15 nM piericidin A have no effect on fusion as compared to control. As is evident from Fig. 3.27, 20 nM piericidin A inhibits CI activity in NSM by 36.3% which is slightly higher than the threshold seen in synaptic mitochondria before major changes in rates of oxygen consumption and ATP synthesis were observed but this may be explained by the presence of mitochondria of glial cell origin in the preparation (Pathak and Davey, 2008). Interestingly, the same concentration of piericidin A reduces CI activity by 54.5% in primary cortical neurons and measurement of the oxygen consumption rate using a seahorse XF24 extracellular flux analyzer shows a decrease of ~50% (Fig. 3.26) indicating a higher than expected threshold for CI activity before mitochondrial fusion is affected. The graphs in Fig 3.28 assume that inhibition of CI in intact cells is the same as the level of inhibition seen when primary neurons have been homogenised and cryolysed to fracture the cell membrane. It is not known the rate at which piericidin A crosses the plasma membrane and

subsequently binds to the site of inhibition on CI. Therefore, it may be more pertinent to compare % inhibition of fusion with % inhibition of respiration as shown by oxygen consumption rates where culture conditions were identical. This inhibition is brought about by a cleavage of Opa1 as demonstrated by an increase in the short form in Fig. 3.29, likely because of the activation of Oma1 by a significant decrease in $\Delta\Psi_m$ after 5 min incubation with 20 nM piericidin A. No effect was seen on ATP levels until cells were treated with concentrations of 100 nM or greater piericidin A.

This type of “tipping point” threshold effect in relation to $\Delta\Psi_m$ has been documented before whereby $\Delta\Psi_m$ has to decrease to 34% of control before mitochondrial fragmentation occurs and a significant decrease in expression of L-Opa1 is detected when the membrane potential is dissipated using increasing concentrations of CCCP (Jones et al., 2017). However, it should be noted that these experiments were carried out in 143b osteosarcoma cells which are likely to be significantly more glycolytic than OXPHOS reliant primary cortical neurons and the levels of reduction in $\Delta\Psi_m$ are much slower than those seen in our cellular models using similar concentrations of an ionophore. Previous studies have shown that rotenone uniformly affects CI throughout the brain but only dopaminergic neurons of the nigrostriatal system show evidence of neurodegeneration suggesting a sensitivity to CI inhibition in this brain region associated with PD (Betarbet et al., 2000). That paper suggested that long term exposure to low dose CI inhibitors may results in a systemic increase in ROS which causes oxidative damage to proteins and DNA over a period of months or years but our research suggests that the CI inhibition may slow/halt mitochondrial fusion once the inhibition reaches a threshold resulting in progressive mitochondrial dysfunction. Opa1 also acts as a metabolic sensor and responds to changes in energetic conditions to regulate cristae structure which subsequently plays a role in super-complex formation (Patten et al., 2014, Cogliati et al., 2013). It is possible that cristae remodelling occurs above the fusion threshold, resulting in super-complex disassembly and less efficient OXPHOS resulting in the progressive neurodegeneration seen in the nigrostriatum. Further

research is required to examine the effect of CI inhibition on super-complex assembly/disassembly.

Ndi1 has shown potential as a therapeutic for several metabolic disorders in the past (Marella et al., 2010, Chadderton et al., 2013) and here we show its potential use for treatment of neurodegenerative disorders associated with mitochondrial dysfunction. Transfection with a humanized form of Ndi1 partially restores fusion rates towards levels seen in control cells and prevents the fragmentation usually seen after treatment with 50 nM piericidin A. Due to poor transfection efficiency we were unable to see if expression of this plasmid prevents cleavage of Opa1 and maintains oxygen consumption rates in the presence of piericidin A but we hypothesize this would be the case considering the restoration of fusion observed. Ndi1 may provide a potential therapeutic in the treatment of neurodegenerative disorders if an efficient method can be found to safely deliver to specific brain regions and express it uniformly throughout these regions. The threshold effect for mitochondrial fusion seen in primary cortical neurons provides further evidence that deficits in mitochondrial fusion may play a role in the pathogenesis of neurodegenerative disorders. It raises the possibility that long term inhibition of CI by protein oligomers, such as α -synuclein, could eventually tip the balance and decrease $\Delta\Psi_m$ sufficiently to inhibit fusion which would result a decrease of bioenergetic function over time, ultimately leading to neuronal cell death.

It was subsequently decided to investigate the effects of known neurotoxins that cause parkinsonism and overexpress α -synuclein to determine if Ndi1 may have a therapeutic role in PD. Since the discovery that the toxin MPP⁺, which is metabolized from MPTP by monoamine oxidase B (MAO-B) in the brain, causes Parkinsonism by inhibition of complex I of the neurons of the substantia nigra (Hala et al., 1983), much of the research on causes of idiopathic PD has shifted to complex I inhibition. MPP⁺ has been shown to damage microtubules, impairing axonal transport and damage mitochondria (Cartelli et al., 2010). It decreases $\Delta\Psi_m$ and intracellular ATP levels, increase ROS production (Piao et al., 2012), and impairs anterograde mitochondrial transport in dopaminergic axons (Kim-Han

and Antenor-Dorsey, 2011). Here we show a direct decrease in mitochondrial fusion 20 min after treatment with a high dose of MPP⁺ in differentiated PC12 cells for the first time (Fig 3.32). It is not known how this effect is mediated but it is likely through a cleavage of Opa1 stimulated after complex I inhibition decreases $\Delta\psi_m$ as has been previously reported (Piao et al., 2012) and shown in Fig 3.17 or a decrease in mitochondrial transport in PC12 cells decreasing the likelihood of a fusion event occurring. The decrease in mitochondrial fusion is not as great as seen with other complex I inhibitors such as rotenone (Fig 3.5) and piericidin A (Fig 3.6) but this is likely due to MPP⁺ more slowly penetrating the hydrophobic reaction site on complex I and because it serves as a substrate for the plasma membrane DA transporter (Storch et al., 2004), meaning its import into the cell is dependent on expression of this transporter which may delay the onset of action. It would be interesting to do a time course over 24 hours or until maximum inhibition of complex I is achieved using a high dose of MPP⁺.

Both α -synuclein aggregation and impaired mitochondrial dynamics have been implicated in the pathogenesis of PD (Irrcher et al., 2010, Büeler, 2009, Baba et al., 1998). In addition to this, overexpression of α -synuclein in SH-SY5Y results in mitochondrial fragmentation through its unique membrane interaction which also has inhibitory effect on vesicle fusion, suggesting mitochondrial fusion and vesicle fusion may be linked. This fragmentation can be rescued by overexpression of WT PD associated genes PINK1, parkin or DJ-1, but not the mutated forms of these genes (Kamp et al., 2010). α -synuclein overexpression can also decrease mitochondrial motility and impact the expression of mitodynamins, such as increasing mitochondrial localization of Drp1 (Xie and Chung, 2012, Gui et al., 2012). Taken together, these results show a clear link between PD and altered mitochondrial dynamics. Here we show, using our real time confocal based mitochondrial fusion assay, that mitochondrial fusion is reduced in differentiated PC12 cells overexpressing WT α -synuclein (Fig 3.33) and mutant (A53T) α -synuclein (Fig 3.34). There is also an obvious change in mitochondrial morphology, where mitochondria are more punctate and rounded indicating that these cells likely have reduced activity of the fusion machinery or

increased localization of Drp1 to the mitochondria as has been reported previously. Other results (unpublished) from the lab have shown that the WT and A53T α -synuclein overexpressing cells show no change in $\Delta\psi_m$ but do have increased ROS production which can directly impact function of the mitodynamins. Different species of α -synuclein oligomers can cause calcium influx into the cell (Danzer et al., 2007) which would increase the activity of calcineurin and directly phosphorylate Drp1, increasing its activity and promoting mitochondrial fragmentation as seen in this study. There is likely to be a wide variety of mitochondrial stimuli, such as complex I inhibition by α -synuclein, influencing mitochondrial morphology in these cells, strengthening the growing link between cellular bioenergetics and mitochondrial dynamics. These data show for the first time that overexpression of α -synculein significantly decreases mitochondrial fusion in differentiated PC12 cells, adding further weight to the hypothesis that PD progression is mediated through malfunctioning mitochondria. It will be interesting to examine if Ndi1 expression in differentiated PC12 cells overexpressing α -synuclein can rescue the deficits of fusion seen in these cells.

Chapter 4

The Effects of Pyruvate and Fatty Acid
Metabolism on Mitochondrial
Dynamics in Differentiated PC12 Cells

4.1 Introduction

Glucose is the primary fuel source for brain cells which is important as the brain requires 20-25% of the total energy produced in the body on any given day. However, under certain conditions, fatty acids, ketone bodies and lactate can be used a fuel source for neurons. In this chapter, we look at the effect of limiting substrate transport into the mitochondrion on fusion dynamics and look at the function of a protein which may link two of the major energy pathways in the mammalian cell; fatty acid oxidation and oxidative phosphorylation.

4.1.1 Nutrient Sources used in the Brain

Most of the glucose consumed by the brain is used to maintain the presynaptic and postsynaptic ion gradients required for neurotransmission while the remainder is used to maintain the resting potential of neurons. Basal rates of energy consumption are high in the awake brain, so any stimulation induced increase is small and localized to a particular brain region (Shulman et al., 2004). The rate limiting step in brain glucose metabolism is believed by some to be the rate of glucose uptake, performed by the glucose transporter GLUT1. This is controversial because GLUT1 is thought to operate at less than half its total transporter capacity (Leybaert et al., 2007). Rate-limiting or not, an isoform of GLUT1 transports glucose across the blood-brain barrier and a second isoform of GLUT1 transports it into astrocytes. Another glucose transporter (GLUT3) transfers the glucose directly into neurons for glycolysis to occur. This appears to be the primary fuel source of neurons but 8-10% is lactate which is shuttled from astrocytes to neurons and metabolized (Magistretti and Allaman, 2015). Once inside the cell, glucose is metabolized to pyruvate which is transported into the mitochondrion by the MPC.

The MPC was first identified as a result of the synthesis of specific inhibitor called α -cyano-4-hydroxycinnamate (CHC), which is an analogue of the *enol* form of pyruvate with an attached aromatic ring. This inhibitor was shown to reduce pyruvate oxidation by rat liver mitochondria but had no effect on pyruvate metabolic enzyme activities,

confirming that transport into the mitochondrion was not through passive diffusion, although there does appear to be some transport in this manner (McCommis and Finck, 2015). UK-5099 was later identified as a more potent inhibitor which inhibits MPC at concentrations of 50 nM and was used for inhibiting pyruvate transport in this study. In recent years, two groups have identified a hetero-oligomeric complex of proteins called MPC1 and MPC2 which are both necessary and sufficient for pyruvate transport into the mitochondrion. Knockdown of either of these genes significantly reduces pyruvate-stimulated mitochondrial respiration and co-expression of murine forms of them in bacteria was sufficient to facilitate pyruvate transport into a bacterium (Daniel et al., 2012, Herzig et al., 2012). The pyruvate transported into the mitochondria is converted to acetyl CoA (and NADH from NAD⁺) by pyruvate dehydrogenase (PDH) and enters the TCA cycle. In neurons, links have been drawn between reduced PDH activity in the context of several neurological disease (Sorbi et al., 1983). A likely explanation for this is reduced pyruvate transport into the mitochondria, subsequently leading to decreased flux into PDH. To assess the effects of pyruvate transport on mitochondrial fission/fusion dynamics in this study pyruvate transport was inhibited using UK-5099 and PDH activity was increased by inhibition of its negative regulator, pyruvate dehydrogenase kinase (PDK), using dichloroacetate (DCA).

It has been widely accepted that neurons do not use fatty acids as an energy source but several recent studies have begun to challenge this dogma. One paper showed isolated rat brain mitochondria utilize fatty acids as an energy source in neurons and astrocytes when administered in association with other respiratory substrates (Panov et al., 2014) while *Drosophila* brains are able to catabolize fatty acids and release ketone bodies (Schulz et al., 2015). Fatty acid transport across the blood brain barrier is a complex process, involving diffusional and protein-mediated transports, predominantly by fatty acid transport protein-1 and -4 (FATP-1/4). Within the brain, fatty acid oxidation for energetic purposes occurs mostly in glial cells, although with a lower efficiency when compared to other tissues with high energy turnover, as CPT1 in the brain has a lower enzymatic capacity (Schönfeld and Reiser, 2013). Most theories argue this lower rate of fatty acid metabolism in the brain is an evolutionary

protective mechanism as fatty acid oxidation results in a higher production of superoxide which would be damaging in neurons particularly vulnerable to oxidative damage. However, there is a subset of neurons who express a specific form of CPT1 called CPT1C which does not support β -oxidation in hypothalamic neurons of arcuate nucleus but does specifically inhibit malonyl-CoA. In mice lacking CPT1C, food intake and body weight is lower than wild-type litter mates but gain excessive body weight and body fat when fed a high fat-diet while maintaining lower or equivalent food intake (Wolfgang et al., 2008). Here we investigate the effect of limiting fatty acid β -oxidation, using etomoxir, and inhibiting malonyl CoA to sustain an increased level of β -oxidation, on mitochondrial dynamics in differentiated PC12 cells as a neuronal model.

4.1.2 Nutrient Sources and Mitochondrial Dynamics

Highly fused mitochondria are evident in most cell types when maximum respiratory capacity is needed as they are more efficient and have increased respiration rates (Westermann, 2012). Thus, it is probable that alterations in the primary nutrient source may have an effect on mitochondrial morphology. Interestingly, beta cells exposed to nutrient excess show increased respiration rates and a robust fragmentation of the mitochondrial network, perhaps as there is more nutrient wastage under such high glucose conditions (Molina et al., 2009). Conversely, low availability of nutrient sources elongates mitochondria by inhibiting Drp1 recruitment to the mitochondria and unopposed fusion occurring. This is associated with an increase in cristae number and ATP synthase dimerization and higher ATP synthase activity, sparing mitochondria from mitophagy (Gomes et al., 2011a). It is evident any changes in total nutrient availability have an effect on mitochondrial dynamics but little is known about how nutrient sources might affect morphology.

Fatty acids are known to provide cellular energy under starved conditions. A study by Rambold et al. (2015) found that fatty acid import into mitochondria under starved conditions was directly from lipid droplets (LDs) to mitochondria and this transfer required mitochondria to be highly fused and localized near LDs. When mitochondrial fusion was prevented in starved cells, fatty acids neither homogeneously distribute

within mitochondria nor became efficiently metabolized. Instead, fatty acids re-associated with LDs and fluxed into neighboring cells. This provides a direct link between fatty acid oxidation and mitochondrial fusion, as stimulation of fusion is required to utilize fatty acids as an energy source. Here, we examine the effect of blocking fatty acid transport into mitochondria on mitochondrial fusion rates when cells are not starved, as one would expect glucose to be the primary nutrient source in this situation. Free fatty acids can be toxic unless their degradation is tightly controlled as they can act as uncouplers and inhibitors of mitochondrial respiration, underlying the importance of tightly controlled fatty acid metabolism in neurons which are susceptible to oxidative damage (Wojtczak and Schönfeld, 1993). LDs have been detected in the axons of *Aplysia* neurons and in cultured neurons and brains sections of Huntington's disease models (Martinez-Vicente et al., 2010). Recent research has linked disrupted LD function with neurodegeneration, including the finding that overexpression of α -synuclein promotes LD accumulation (Outeiro and Lindquist, 2003). These data suggest that disruptions in fatty acid metabolism may play a role in the alterations in mitochondrial dynamics seen in neurodegenerative disease.

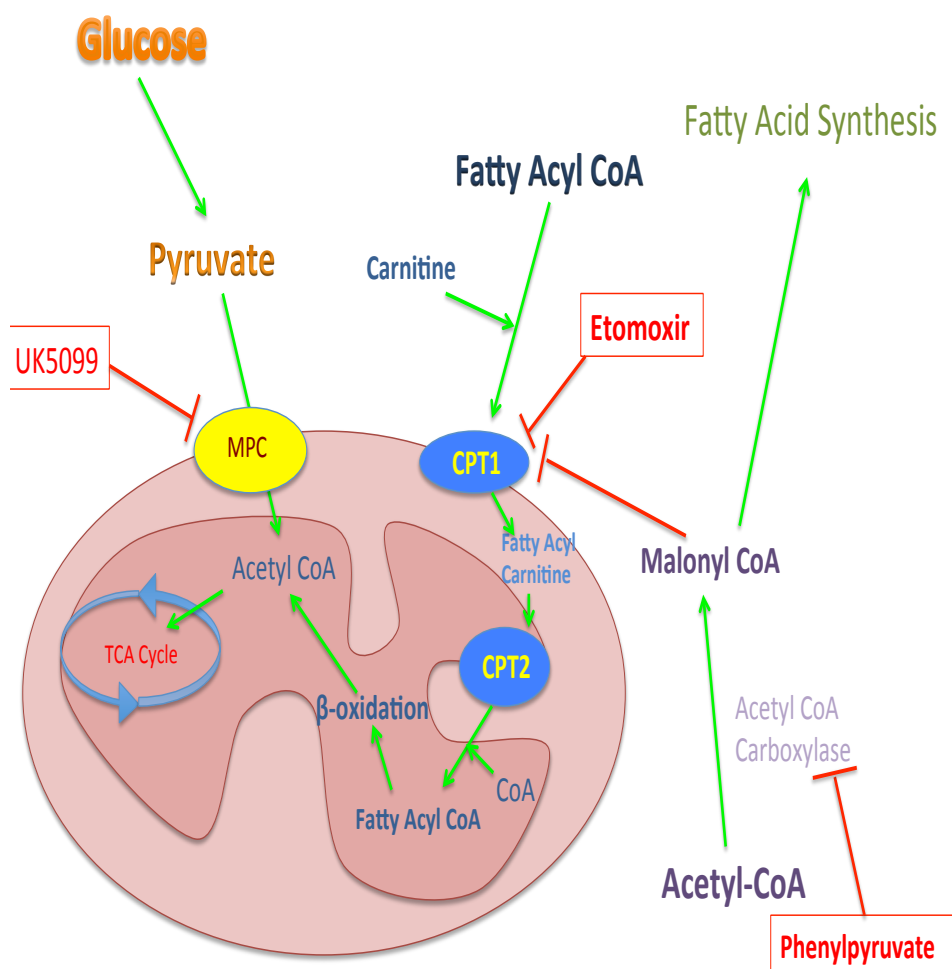


Figure 4.1 – Mechanisms of Substrate Transport into the Mitochondrion. Figure showing how the transport of pyruvate into mitochondria is mediated by the mitochondrial pyruvate carrier (MPC), a complex of 150kDa with an unknown of protein subunits. Fatty Acyl CoA enters the mitochondrion through the carnitine system, the rate limiting step of which is transport across the outer membrane by carnitine palmitoyl transferase 1 (CPT1). Both substrates are metabolized and enter the TCA cycle.

4.1.3 Aims of the Chapter

- To examine the effect of inhibition of fatty acid oxidation on mitochondrial dynamics in differentiated PC12 cells
- To investigate the effect of inhibition of pyruvate transport into the mitochondrion on mitochondrial dynamics in differentiated PC12 cells
- To knockdown ACAD9 in differentiated PC12 cells and examine the effect of this on mitochondrial function

4.2 Methods

4.2.1 Generation of Stable ACAD9 knockdown PC12 cells

PC12 cells were seeded in a 96 well plate at a density of 2.0×10^4 cells/well in fresh media and were incubated overnight in a humidified incubator at 37 °C, 5% CO₂. The following day, media was changed and based on the optimized multiplicity of infection (MOI) of 20 for PC12 cells, lentiviral particles (4 constructs) were added to each well and incubated at 37 °C overnight. After 24 hours, media containing lentiviral particles was removed and cells were maintained in normal RPMI 1640 (10% FBS, 1% P/S), the following day, media was replaced with fresh media containing 800ng/ml puromycin to begin the selection process. After 3-4 days, 3 resistant colonies for each construct were selected and expanded to be assayed for total ACAD9 protein levels by western blot. The knockdown was confirmed by qRT-PCR and the cells with the largest reduction in relative mRNA expression were expanded, frozen down and used for future experiments. The constructs were designed by Sigma Aldrich using the Rosetta algorithm and siRNA sequences were as follows.

ACAD9-1 GTTCTGCGTGGAAGCTTATTT

ACAD9-2 GAGACCCTGTTACTCCGATTT

ACAD9-3 ATTCAGGTCCCAGAAGAATAT

ACAD9-4 CCCAGCTGACACCTTAGCGAA

4.2.2 RNA Extraction

Total RNA was extracted from differentiated PC12 cells 48 hours after differentiation using the RNeasy Mini kit from Qiagen.

4.2.3 cDNA Synthesis

First strand cDNA was created by reverse transcription from the total RNA using the Qiagen RT2 first strand kit. Briefly, 1 µg RNA was added to 6 µl of genomic DNA elimination buffer and the final volume was adjusted to 14 µl using RNase-free H₂O.

The samples were incubated at 37 °C for 5 min and immediately placed on ice for 1 min. following this, 6 µl of the BC5 reverse transcriptase mix was added to each sample and incubated at 42°C for exactly 15 min. The reaction was then stopped by heating to 95°C for 5 min. The completed cDNA was stored at -20°C until ready for qRT-PCR analysis.

4.2.4 Quantitative Real-time Polymerase Chain Reaction

Relative quantification of the transcript was performed using the Brilliant II SYBR ® green qRT-PCR one step kit and the MX3000P from Stratagene. RNA was isolated from cells as described in section 4.2.1. Primer sets were designed using the MWG online primer design tool and were optimized for the RT and amplification steps (Table 2.1). Each pair was also assessed to ensure > 95% efficiency during amplification stages. The transcript levels were normalized to the GAPDH transcript level, which is a known housekeeping gene of constant expression level.

Name	Sequence	Gene
ACAD9_For	TGGGCTCAAGAATCACGACC	ACAD9
ACAD9_Rev	CAGGTTCTCTGGAGCATACTTGT	ACAD9
GAPDH_For	AACCTGCCAAGTATGATGAC	GAPDH
GAPDH_Rev	GGAGTTGCTGTTGAAGTCA	GAPDH

4.3 Results

4.3.1 Inhibition of CPT1 or the MPC Reduces Mitochondrial Fusion in Differentiated PC12 cells

Inhibition of CPT1 using etomoxir resulted in a dose dependent inhibition of mitochondrial fusion in differentiated PC12 cells. As seen in Fig 5.1, low dose etomoxir treatment (1 μ M) has no effect on fusion compared to untreated control. However, 1 hour pre-incubation with 100 μ M or 1 mM etomoxir resulted in a dose dependent decrease in mitochondrial fusion (Fig 4.3 and 4.4). The higher concentration resulted in ~80% decrease in loss of PA-GFP signal 45 min after activation which implies fatty acid oxidation is a major source of nutrients for PC12 cells or there may be some toxicity associated with high concentrations of the drug. As shown by Merrill et al. (2002), treatment of HepG2 cells with 1 mM etomoxir, resulted significant modulations in the expression of several redox related and cell cycle mRNAs as measured by microarray analysis.

Inhibition of the MPC with UK5099 as shown in Fig 4.5, resulted in a significant decrease in mitochondrial fusion. The concentration used should block pyruvate transport but there may be some free diffusion across the mitochondrial membrane and amino acid metabolism may contribute to maintenance of some level of ATP production. When UK5099 and etomoxir were pre-incubated for 1 hour prior to beginning the fusion experiment, there was a combined effect and mitochondrial fusion was almost completely inhibited (Fig 4.6). These data suggest that fatty acid oxidation supplies a significant portion of acetyl-CoA for the TCA cycle, and when both pyruvate transport and fatty acid oxidation are inhibited, the cell cannot maintain mitochondrial fusion.

Finally, as seen in Fig 4.7, inhibition of acetyl CoA carboxylase results in a significant decrease in mitochondrial fusion. Acetyl CoA carboxylase catalyzes the conversion of acetyl CoA to malonyl CoA and so by inhibiting it, one would expect to increase the activity of CPT1 and thus increase fatty acid oxidation, potentially increasing mitochondrial fusion. However, phenylpyruvate acts on a wide variety of targets and

at the concentration used (20 mM) it is likely the inhibition of fusion seen is as a result of this toxicity.

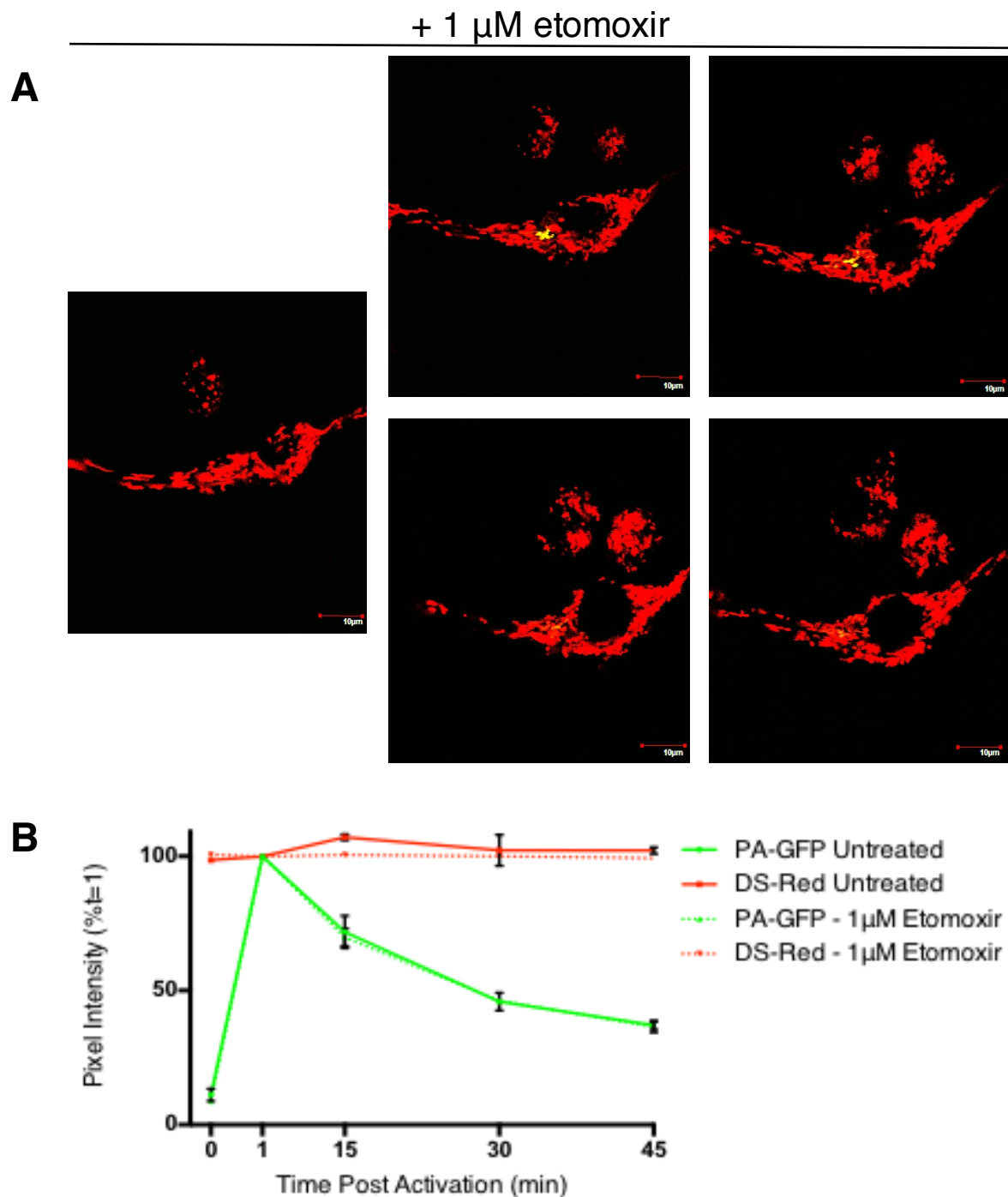


Figure 4.2: Inhibition of carnitine palmitoyl transferase 1 with low concentrations of etomoxir has no effect on mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 1 hour pre-incubation with 1 μ M etomoxir on mitochondrial fusion. (B) Quantitative data shows no inhibition of fusion in etomoxir treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, n=3. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

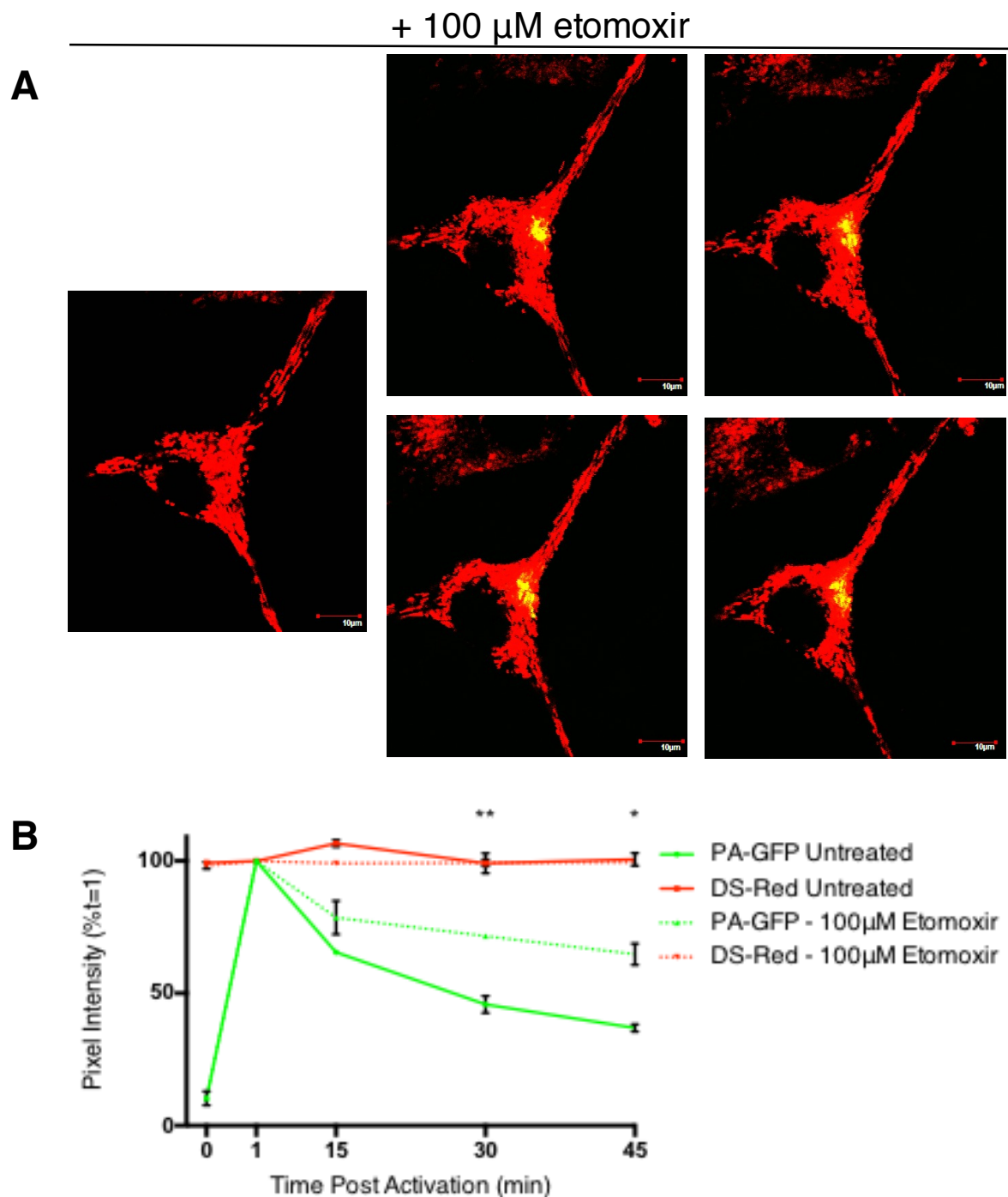


Figure 4.3: Inhibition of carnitine palmitoyl transferase 1 with etomoxir reduces mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 1 hour pre-incubation with 100 μ M etomoxir on mitochondrial fusion. (B) Quantitative data shows inhibition of fusion in etomoxir treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, n=3. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

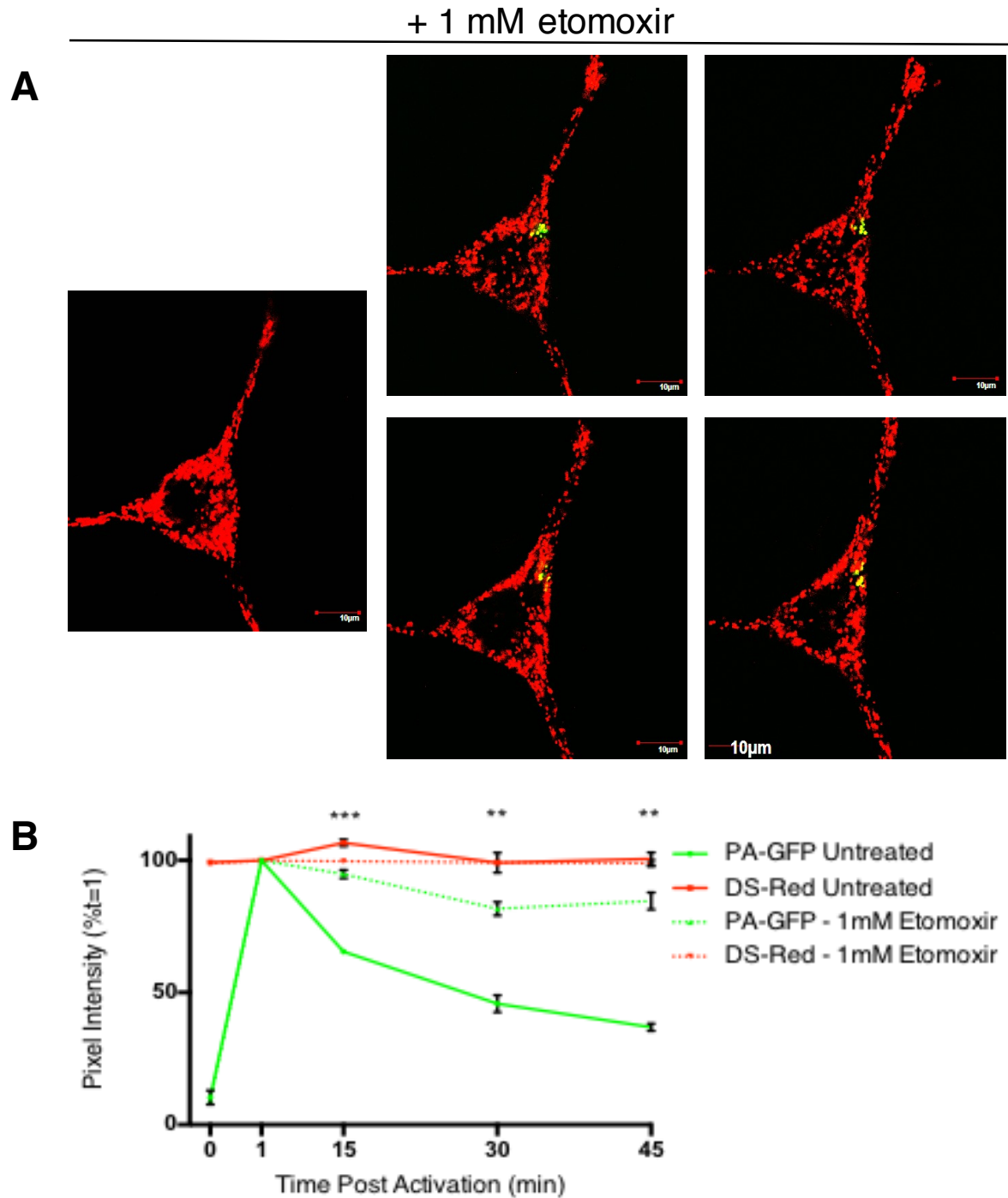


Figure 4.4: Inhibition of carnitine palmitoyl transferase 1 with etomoxir reduces mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 1 hour pre-incubation with 1 mM etomoxir on mitochondrial fusion. (B) Quantitative data shows inhibition of fusion in etomoxir treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

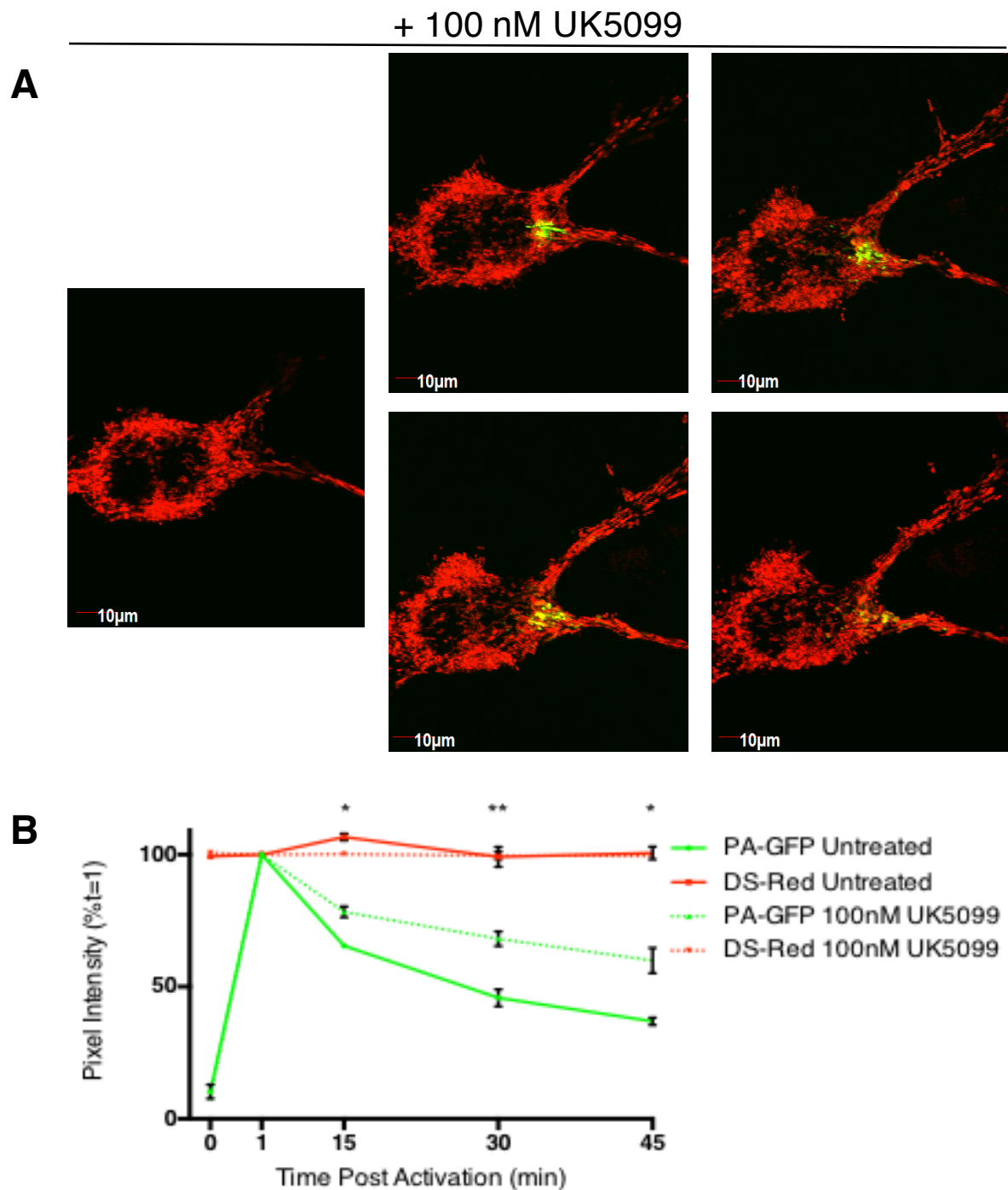
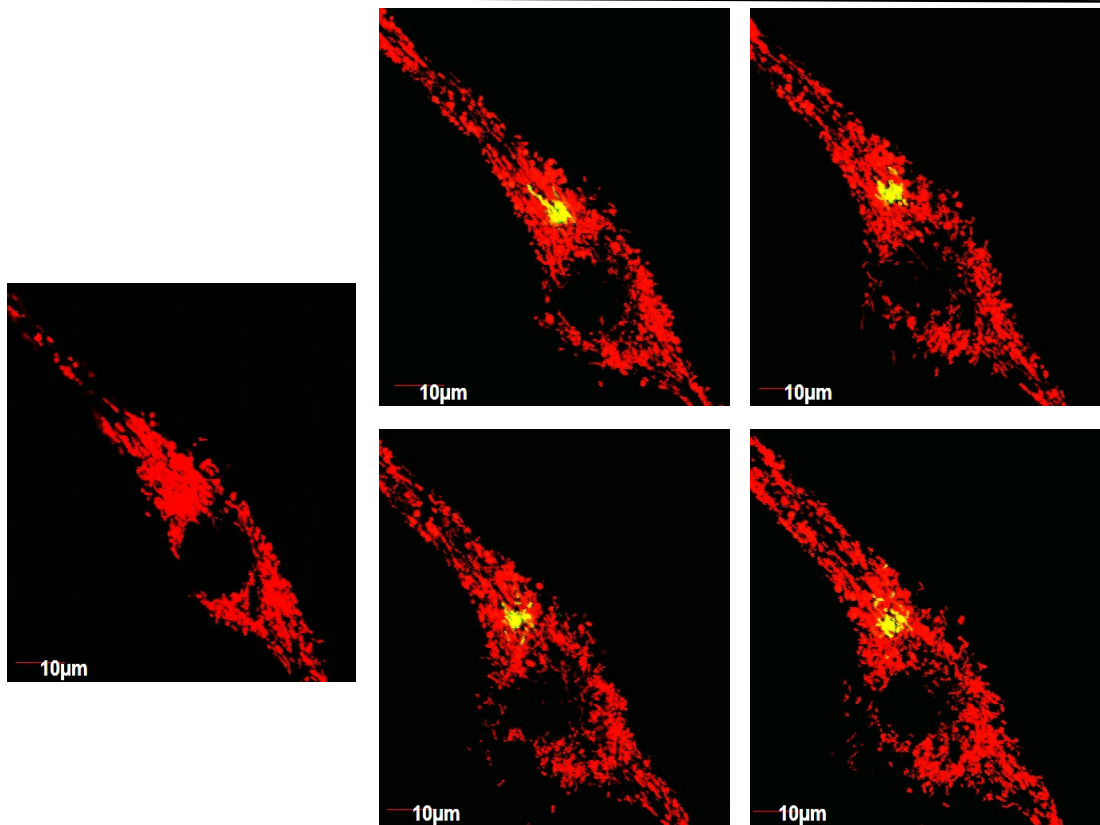


Figure 4.5: Inhibition of the mitochondrial pyruvate carrier with UK5099 reduces mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 1 hour pre-incubation with 100 nM UK5099 on mitochondrial fusion. (B) Quantitative data shows inhibition of fusion in UK5099 treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

+ 100 nM UK5099 and 100 μ M etomoxir

A



B

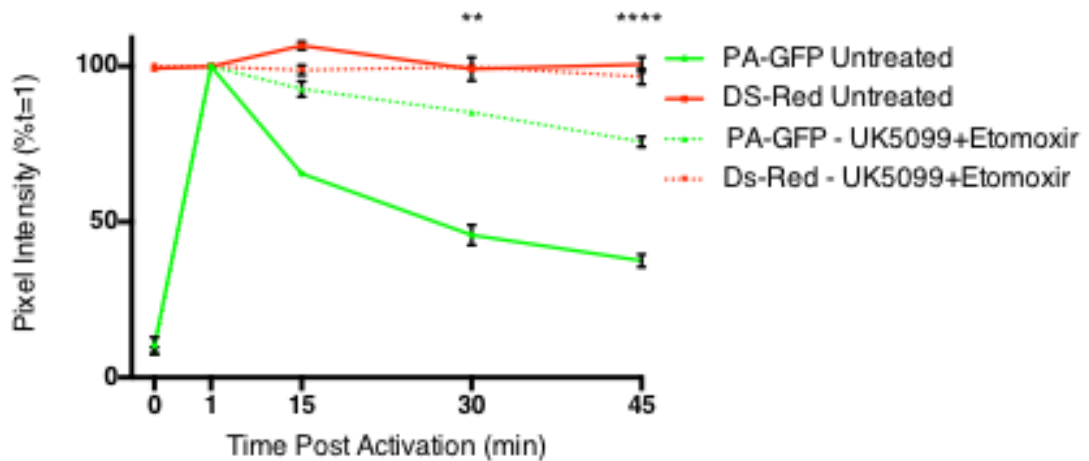


Figure 4.6: Inhibition of carnitine palmitoyl transferase 1 and the mitochondrial pyruvate carrier with etomoxir and UK5099 reduces mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 1 hour pre-incubation with 100 μ M etomoxir and 100 nM UK5099 on mitochondrial fusion. (B) Quantitative data shows inhibition of fusion in etomoxir and UK5099 treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

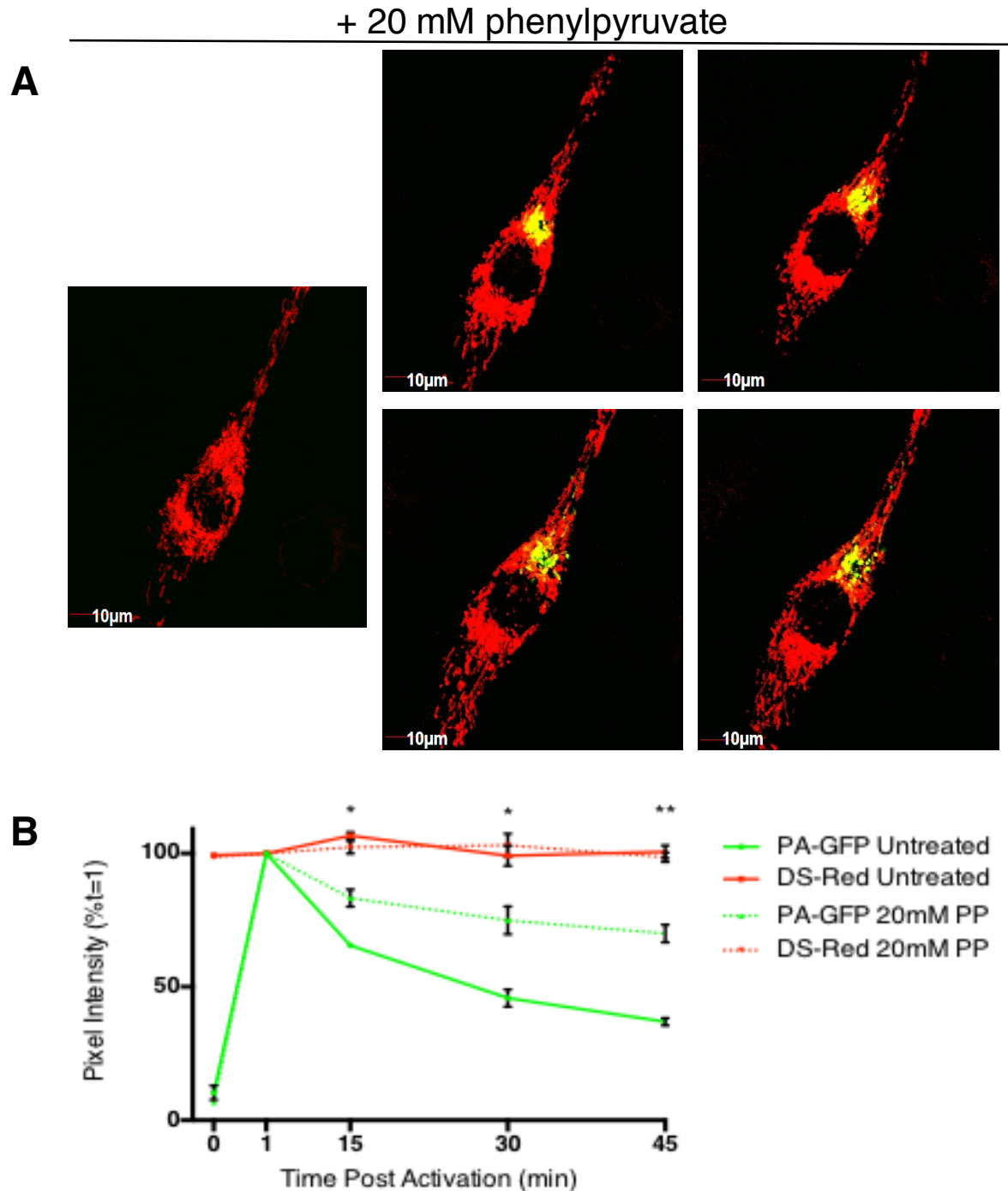


Figure 4.7: Inhibition of acetyl CoA carboxylase with sodium phenylpyruvate reduces mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 1 hour pre-incubation with 20 mM sodium phenylpyruvate on mitochondrial fusion. (B) Quantitative data shows inhibition of fusion in phenylpyruvate treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

4.3.2 Inhibition of Pyruvate Dehydrogenase Kinase Overcomes the Inhibitory Effects of etomoxir on Mitochondrial Fusion in Differentiated PC12 cells

Dichloroacetate is a small molecule that inhibits PDK, thus activating PDH and so increase the flux of pyruvate into the mitochondria. In Fig 4.8, it is shown that inhibition of PDK has no effect on mitochondrial fusion in PC12 cells, indicating that pyruvate transport into the mitochondrion is already sufficient to ensure the maximum rate of mitochondrial fusion. When fatty acid oxidation is inhibited with etomoxir, mitochondrial fusion is decreased (Fig 4.3, 4.4) but co-incubation with DCA allows the cell to overcome the decrease in fatty acid oxidation by increasing pyruvate flux into the mitochondrion and generating more acetyl CoA through this pathway (Fig 4.9). These results show the adaptability of PC12 cells to nutrient sources and allow them to maintain normal mitochondrial fusion.

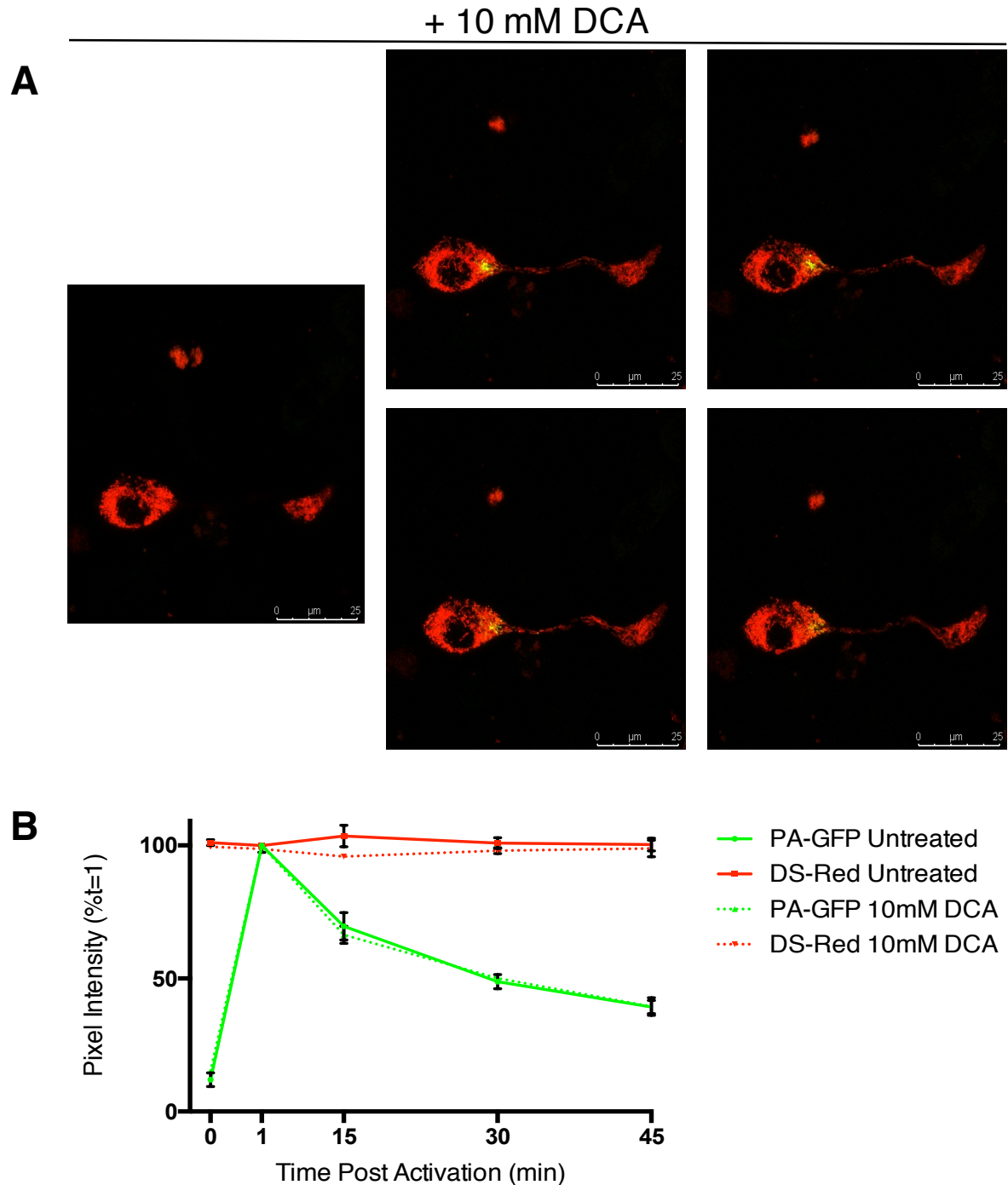
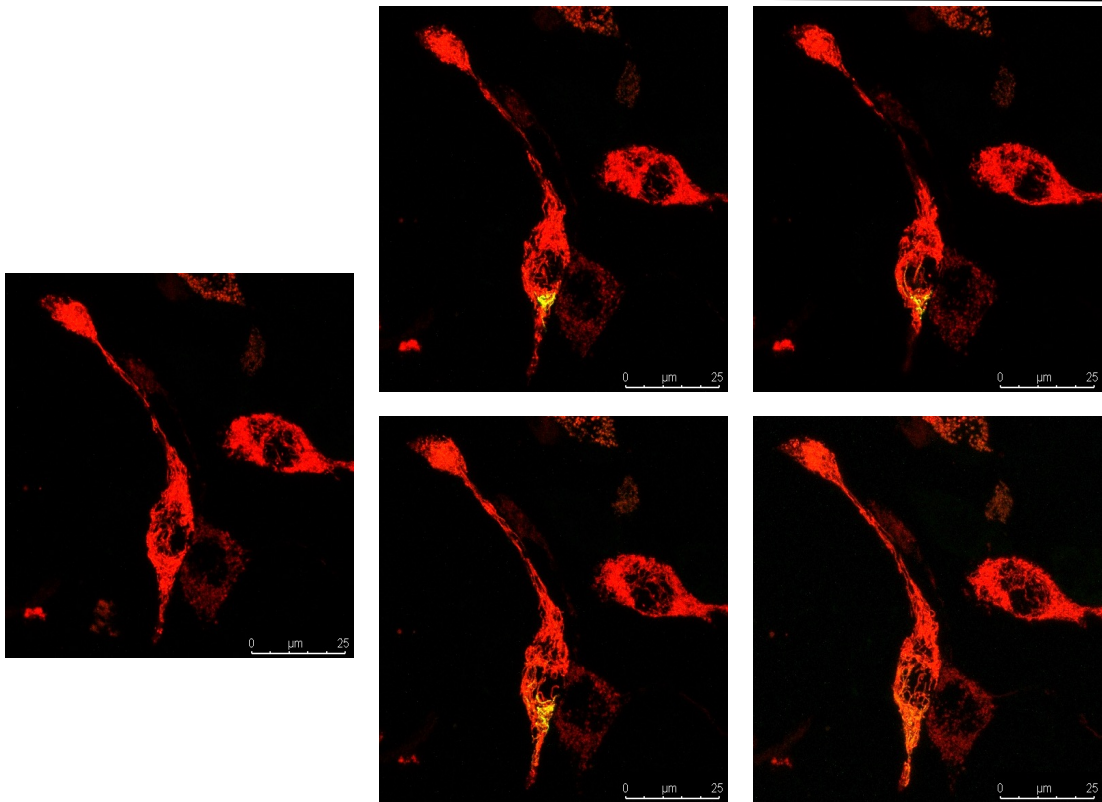


Figure 4.8: Inhibition of pyruvate dehydrogenase kinase with dichloroacetate has no effect on mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 1 hour pre-incubation with 10 mM dichloroacetate on mitochondrial fusion. (B) Quantitative data shows no inhibition of fusion in dichloroacetate treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

+ 100 μ M etomoxir and 10 mM DCA

A



B

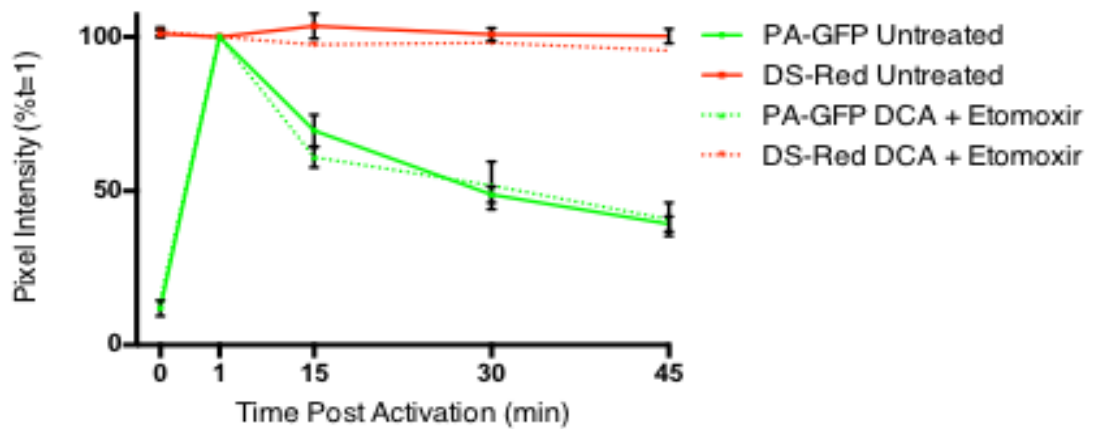


Figure 4.9: Inhibition of pyruvate dehydrogenase kinase with dichloroacetate abolishes the inhibitory effect of etomoxir on mitochondrial fusion in differentiated PC12 cells.

(A) Images representative of the effect of a 1 hour pre-incubation with 10 mM dichloroacetate and 100 μ M etomoxir on mitochondrial fusion. (B) Quantitative data shows no inhibition of fusion in dichloroacetate and etomoxir treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, n=3. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

4.3.3 Differentiation of PC12 cells Decreases Complex I Subunit Expression

Differentiated PC12 cells would be expected to rely heavily on OXPHOS for energy production as they are a post-mitotic cell line. Interestingly, as shown in Fig 4.10, expression of complex I assembly factor ACAD9 and complex I subunit NDUF9 are both decreased in differentiated PC12 cells. This result is somewhat surprising considering the larger size of differentiated PC12 cells but the decreased in complex I protein may be complemented by an increase in complex I specific activity.

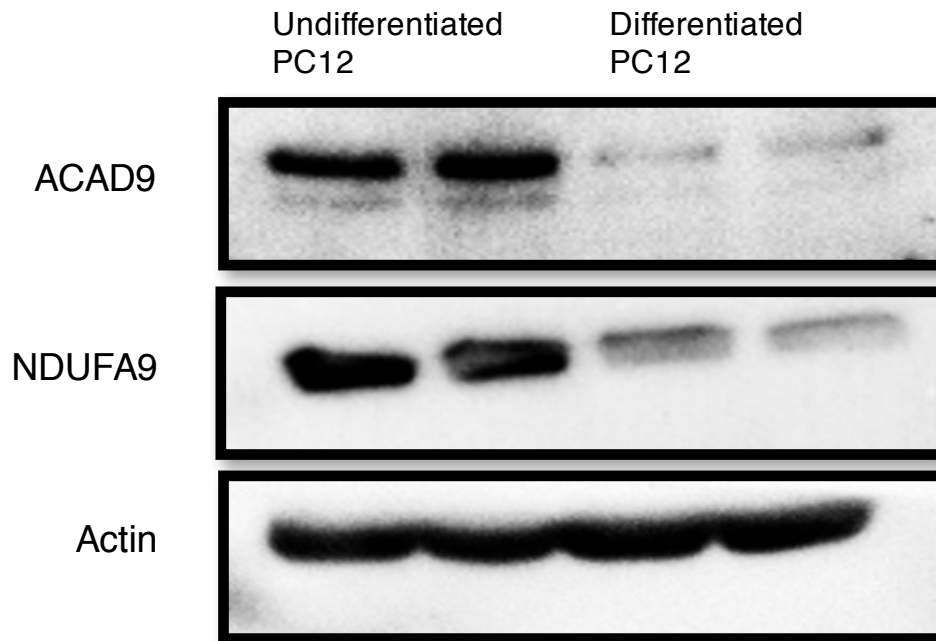


Figure 4.10: ACAD9 expression is reduced in differentiated PC12 cells compared to undifferentiated PC12 cells. PC12 cells were seeded on poly-d-lysine coated dishes and differentiated using NGF (lanes 3 and 4) or grown in normal RPMI 1640 supplemented with 10% FBS (lanes 1 and 2) before being lysed with RIPA buffer and analysed for ACAD9 and NDUFA9 by western blot. Reduced protein levels of ACAD9 and complex I subunit NDUFA9 were observed in the differentiated cells. Image representative of n=2.

4.3.4 Knockdown of ACAD9 in PC12 cells

As a result of the decrease in ACAD9 expression in differentiated PC12 cells, the effect of etomoxir on mitochondrial fusion and previous data from the lab identifying ACAD9 as having a potential role in neuronal differentiation, it was decided to knockdown ACAD9 and examine its effect on mitochondrial function. As seen in Fig 4.11, knockdown using 4 different constructs yield varying levels of reduction in protein expression. From these results, it was decided construct ACAD9 KD1 resulted in the greatest reduction in protein expression. This result was confirmed when mRNA levels were assayed using qRT-PCR as shown in Fig 4.12.

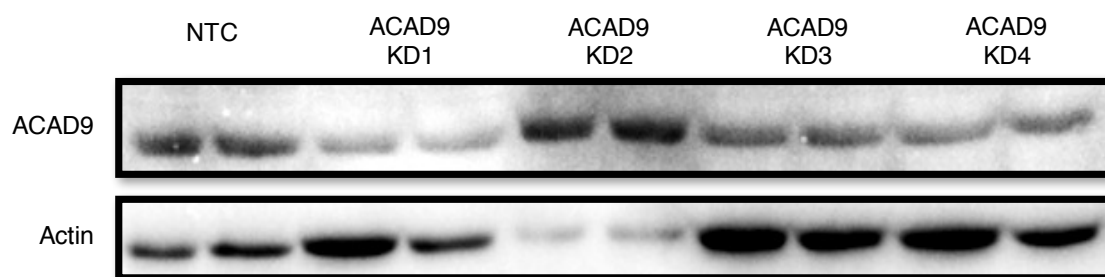


Figure 4.11: Optimization of ACAD9 knockdown in differentiated PC2 cells. PC12 cells were transduced with lentiviral constructs to knockdown ACAD9, single colonies were chosen and expanded. Once grown cells were seeded on poly-d-lysine coated dishes and differentiated using NGF for 48 hours before being lysed with RIPA buffer and analysed for ACAD9 by western blot. Reduced protein levels of ACAD9 were observed in the transduced cells but construct KD1 was termed to achieve the highest level of knockdown. Image representative of n=2.

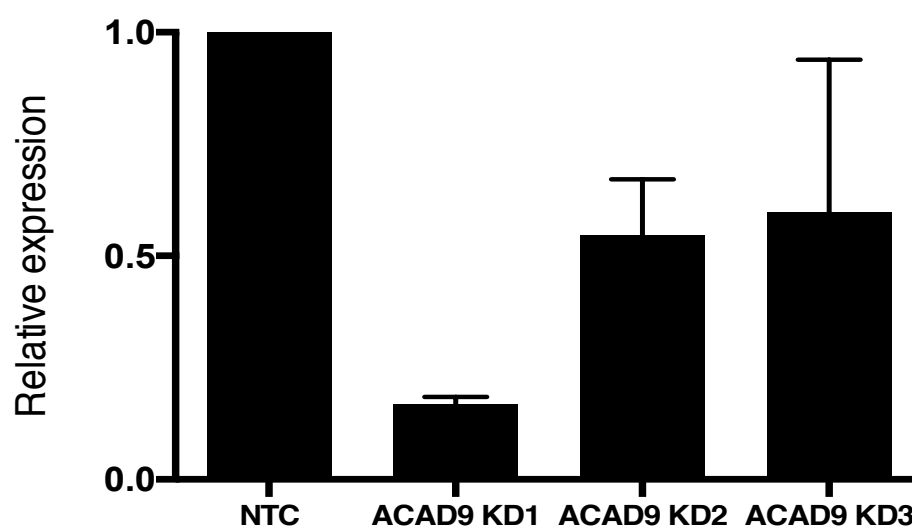
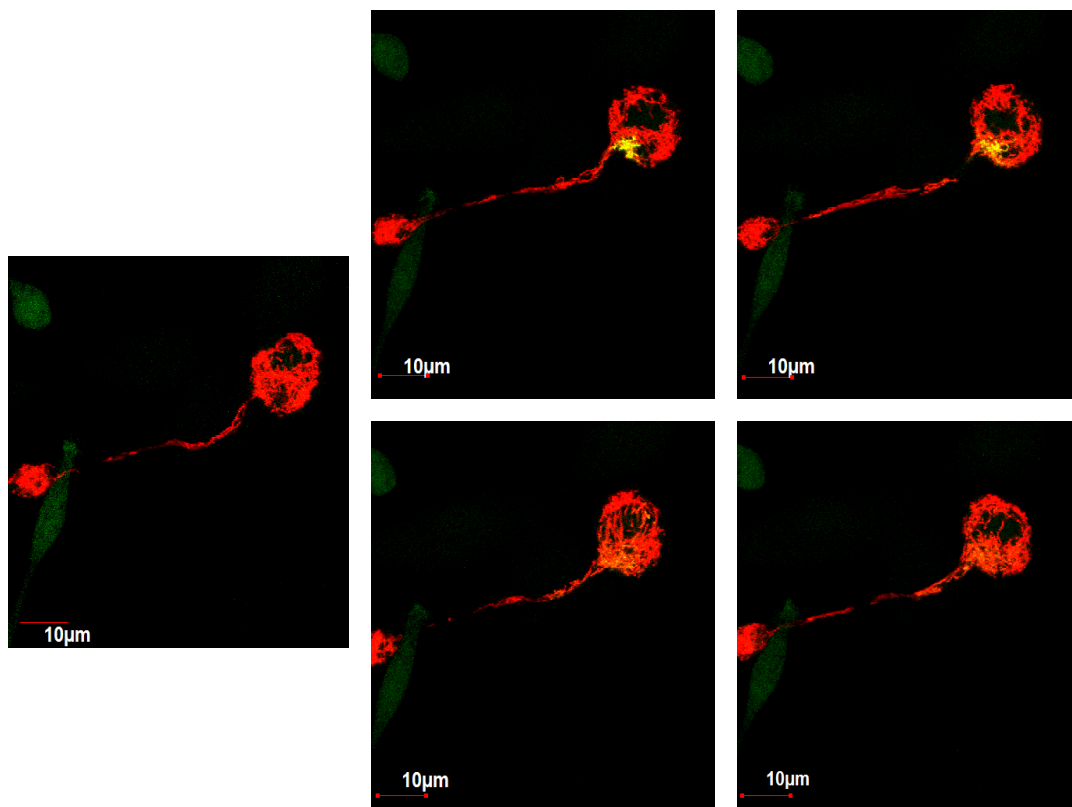


Figure 4.12: ACAD9 mRNA is reduced in knockdown cells. Scramble (NTC) and knockdown PC12 cells were seeded on a poly-d-lysine coated 6 well plate and differentiated for 48 hours before being washed, scraped and RNA was extracted using a Qiagen RNeasy mini kit. The relative level of expression of ACAD9 mRNA was estimated by quantitative reverse transcriptase PCR (qRT-PCR) using the Brilliant ® SYBR green qRT-PCR Master Mix Kit (1-step) from Agilent, in an MxPro 3000 instrument (Stratagene), as described in the methods section. The levels of ACAD9 mRNA were normalized against GAPDH mRNA and the relative quantification was calculated by the $\Delta\Delta CT$ method and expressed as a ratio of non-target control to knockdown. Data expressed as mean $\pm$ SEM, n=3.

4.3.5 Effect of ACAD9 knockdown on Mitochondrial Fusion, $\Delta\psi_m$ and ROS Production in Differentiated PC12 cells

Previous data from the lab has indicated that knockdown of ACAD9 decreases neurite formation in induced pluripotent stem (iPS) cells differentiated to neurons. There was no obvious effect on differentiation of PC12 cells so it was decided to assay mitochondrial fusion to see if a decrease in a complex I assembly factor/ component of the fatty acid oxidation pathway would have an effect on mitochondrial fusion. As shown in Fig 4.13, the NTC construct had the same levels of mitochondrial fusion as untransduced control cells meaning the lentiviral constructs do not affect mitochondrial fusion. Knockdown of ACAD9 also had no effect on mitochondrial fusion as compared to NTC (Fig 4.14). There was a slight, non-significant decrease in $\Delta\psi_m$ (Fig 4.15), while no changes were observed in ROS production (Fig 4.16). In addition to this, Fig 4.17 shows no significant differences were observed in ROS production when OXPHOS was inhibited in ACAD9 knockdown cells as compared to NTC cells. Overall, these data show that ACAD9 knockdown does not appear to have any significant disruption to mitochondrial function in differentiated PC12 cells but further bioenergetic experiments are required to confirm this is the case.

A



B

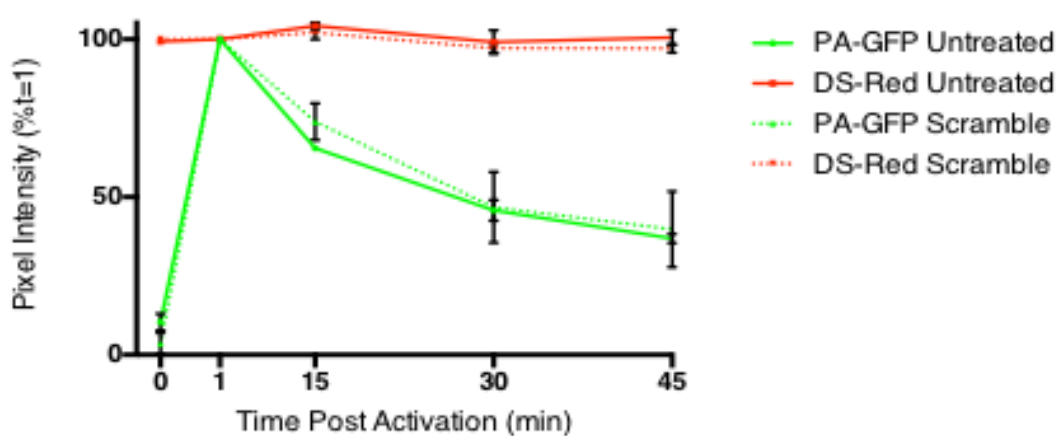
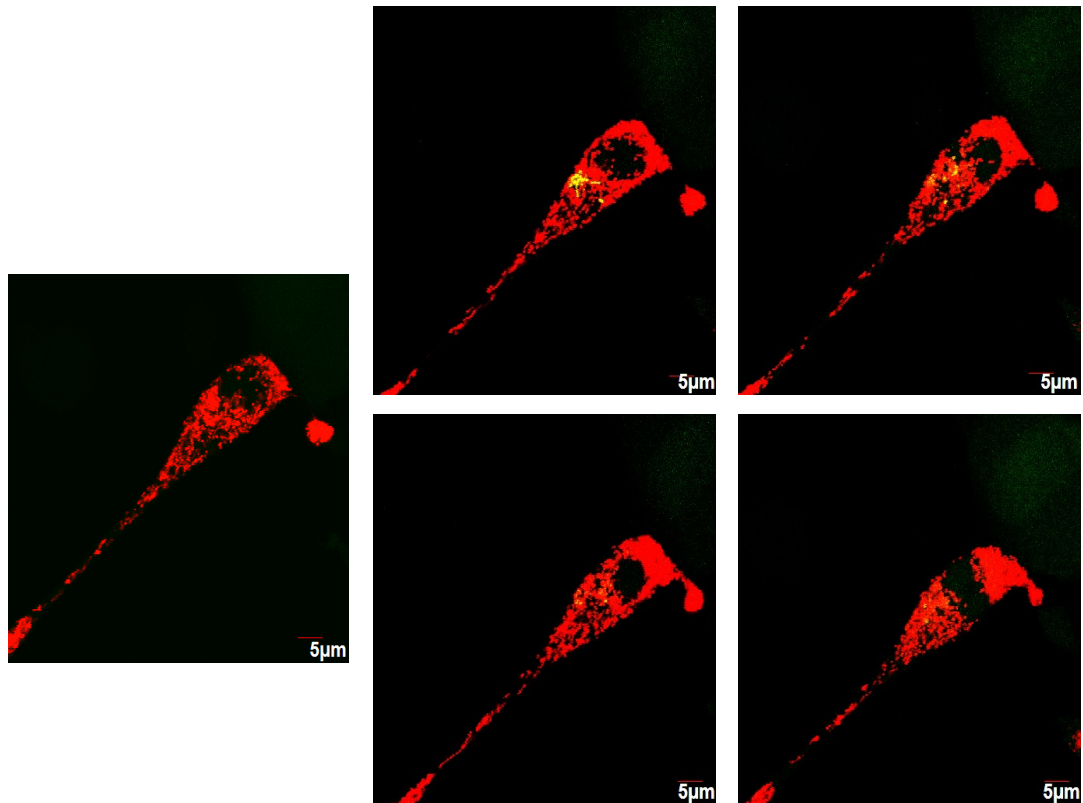


Figure 4.13: Differentiated PC12 cells transduced with non-target shRNA (Scramble) have the same mitochondrial fusion as untransduced controls. (A) Images representative of cells transduced with non-target control shRNA and transfected with DS-Red2 and PA-GFP mito before being analysed for mitochondrial fusion. (B) Quantitative data shows no inhibition of fusion in non-target shRNA transduced cells (dashed green line) compared to untransduced controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

A



B

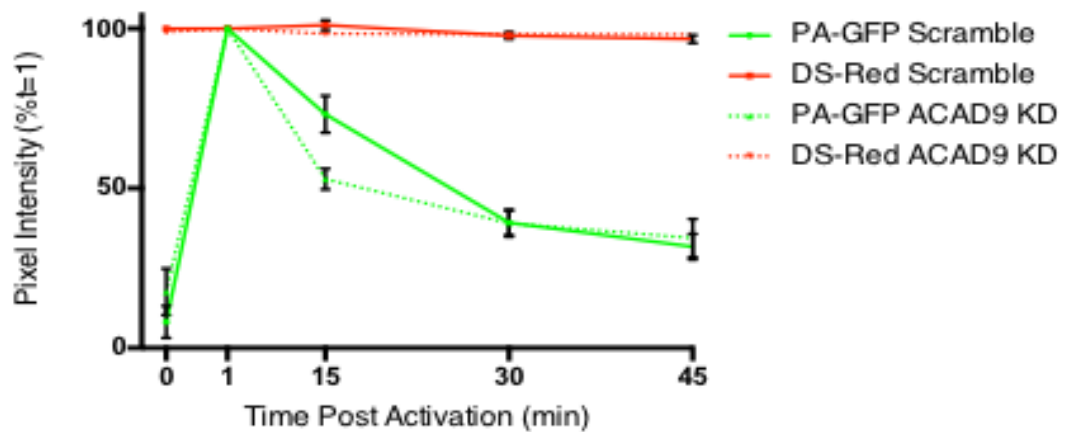


Figure 4.14: Knockdown of ACAD9 has no effect on mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of ACAD9 knockdown on mitochondrial fusion. (B) Quantitative data shows no inhibition of fusion in cells in which ACAD9 is knocked down (dashed green line) compared to non-target controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

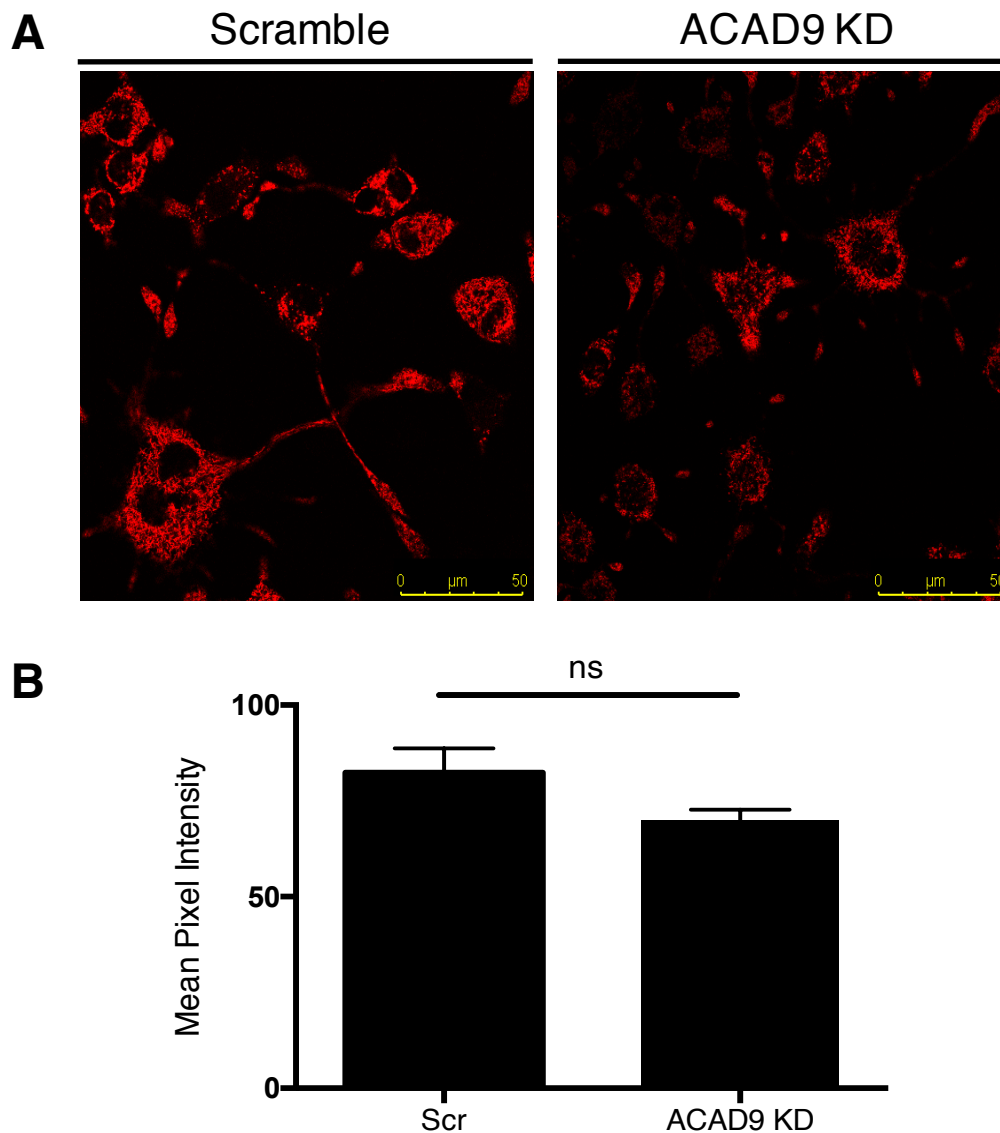


Figure 4.15: Knockdown of ACAD9 has no effect on mitochondrial membrane potential in differentiated PC12 cells. (A) Images representative of TMRM fluorescence in scramble and ACAD9 knockdown cells. (B) Analysis of pixel intensity using imaris image analysis software shows no significant difference in pixel intensity as a measure of $\Delta\psi_m$. Data presented as mean $\pm$ SEM; n=3. A students t-test was performed and values which are significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

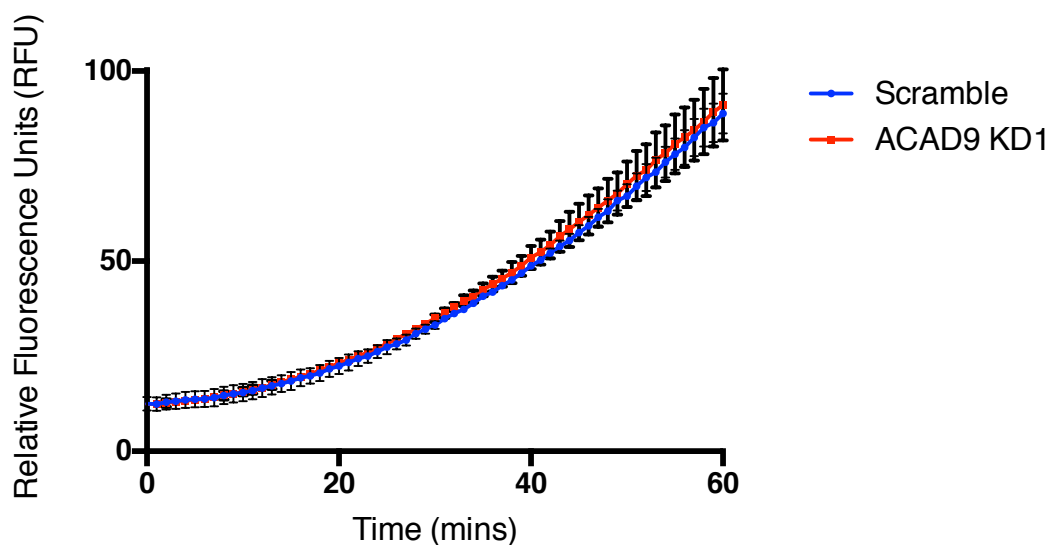


Figure 4.16: Knockdown of ACAD9 has no effect on ROS production in differentiated PC12 cells. Differentiated PC12 scramble and ACAD9 knockdown cells were seeded in a black 96 well plate and differentiated for 48 hours before ROS production was measured using H₂DCFDA. Cells were washed with PBS and maintained in krebs buffer containing 5 μ M H₂DCFDA while fluorescence (excitation λ =485 and emission λ =530) was measured for 1 hour. ROS production was not significantly altered in ACAD9 knockdown cells. Data is expressed as relative fluorescence units $\pm$ SEM; n=3. An individual t-test was performed at each time point and values that are significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

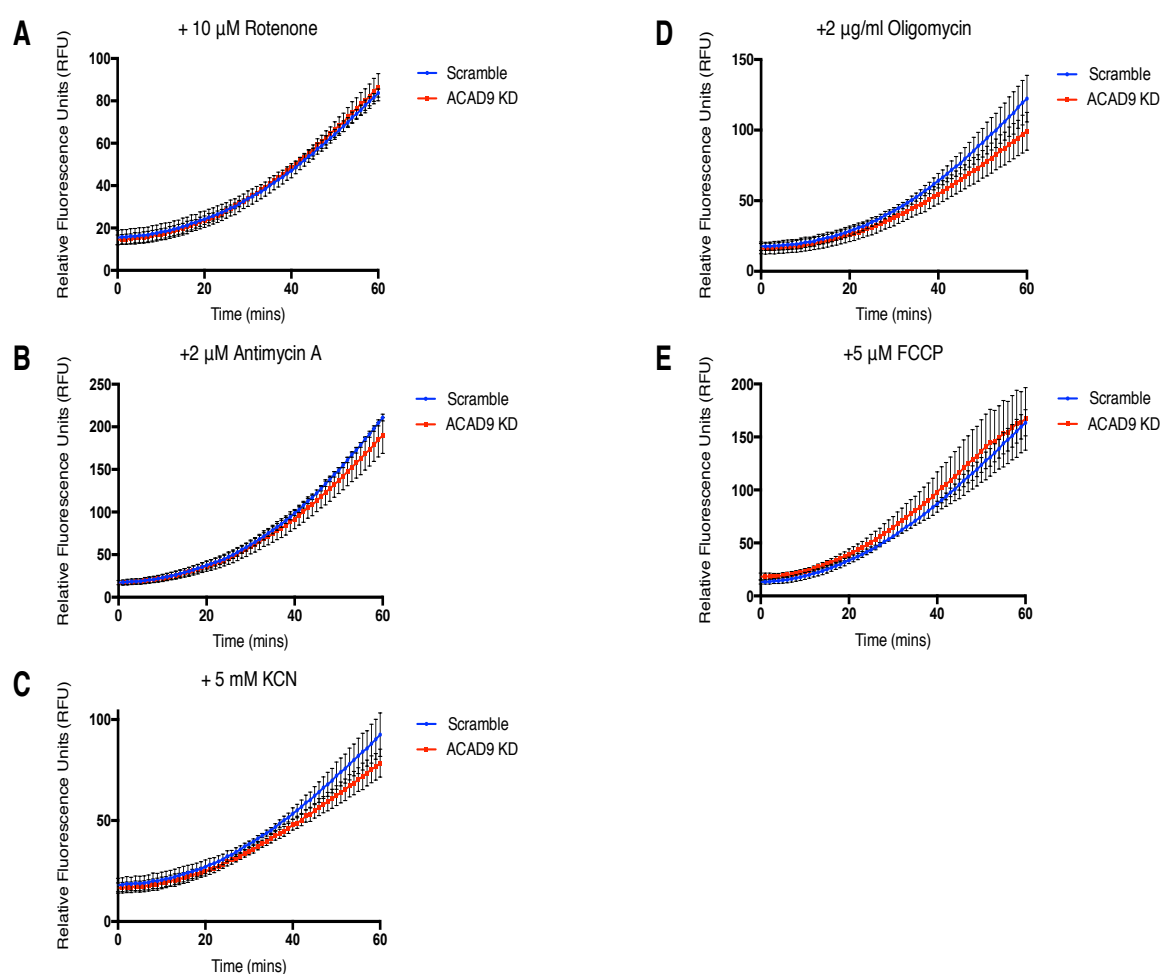


Figure 4.17: Inhibition of OXPHOS in ACAD9 knockdown cells has no significant effect on ROS production. Differentiated PC12 scramble and ACAD9 knockdown cells were seeded in a black 96 well plate and differentiated for 48 hours before ROS production was measured using H₂DCFDA. Cells were washed with PBS and maintained in krebs buffer containing 5 μ M H₂DCFDA and incubated with (A) 10 μ M rotenone, (B) 2 μ M antimycin A, (C) 5 mM potassium cyanide, (D) 2 μ g/ml oligomycin or (E) 5 μ M FCCP and fluorescence (excitation λ =485 and emission λ =530) was measured for 1 hour. There was no significant difference in ROS production in ACAD9 knockdown cells compared to scramble although complex III inhibition did significantly increase the rate of ROS production in both knockdown and scramble cells. Data is expressed as mean relative fluorescence units $\pm$ SEM; n=3. An individual t-test was performed at each time point and values that are significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

4.4 Discussion

Little research has been carried out into the effect of substrate transport or nutrient availability on mitochondrial dynamics except in cases of starvation or high glucose treatment. This is somewhat surprising considering fatty acid oxidation and amino acid metabolism can significantly contribute to cellular metabolism in most tissues of the body. In particular the brain is thought to use glucose, and lactate from neighboring glial cells, almost exclusively as a substrate for ATP production but recent research has suggested that certain subsets of neurons may use fatty acid oxidation as a nutrient source under certain conditions (Panov et al., 2014, Schulz et al., 2015). In spite of this, the neuron specific form of CPT1, (CPT1c) has been shown to have negligible catalytic activity in comparison to other isoforms and instead plays a role in hypothalamic control of energy homeostasis and hippocampal dependent spatial learning (Carrasco et al., 2013, Wolfgang et al., 2006).

This study found that inhibition of CPT1 by etomoxir in differentiated PC12 cells resulted in a significant reduction in the rate of mitochondrial fusion. It was also found that this effect was dose dependent as there was a much more pronounced inhibition of fusion after treatment with 1mM etomoxir (Fig 4.4) when compared to 100 μ M treatment (Fig 4.3) and no inhibition was observed with a 1 μ M treatment (Fig 4.2). This suggests differentiated PC12 cells use the β -oxidation of fatty acids as an energy source to drive mitochondrial fusion. Indeed, some evidence has suggested that about 20% of the total energy expenses of the adult brain are satisfied by oxidation of fatty acids but much of this occurs in astrocytes and so would not explain the large reduction in fusion shown here (Ebert et al., 2003). However, recent research by Panov et al. (2014) sheds more light on the ability of neurons to use fatty acids as an energy substrate. This group isolated synaptic mitochondria from rats and found that incubation with palmitoyl carnitine alone resulted in a higher level of state 4 respiration than incubation with glutamate or pyruvate and that palmitoyl carnitine also showed significant state 3 respiration rates although not as high as glutamate or pyruvate. Of particular interest was the

fact that simultaneous incubation of carnitine palmitoyl with pyruvate, glutamate or succinate resulted in increased state 3 respiration and a several-fold increase in the generation of ROS. In agreement with this, Stoll et al. (2015) showed that neural stem/progenitor cells (NSPCs) demonstrate sustained decreases in oxygen consumption upon treatment with etomoxir. This provides evidence that neurons use a variety of energy sources, most likely due to their high energy demand, and inhibition of one of these (such as fatty acid oxidation) could have a significant effect on neuronal respiration rates and subsequently, mitochondrial fusion.

Even accounting for β -oxidation of fatty acids as an energy source for PC12 cells, it seems unlikely that it contributes enough to account for the 70% reduction in fusion rates observed with the 1mM etomoxir treatment. However, etomoxir treatment has been shown to elicit a variety of other mitochondrial effects. Lee et al. (2014) found that inhibition of CPT1 by etomoxir caused an increase in ROS production in INS-1 beta cells, Pike et al. (2011) showed that etomoxir reduced cellular ATP levels, NADPH levels and increased intracellular ROS in glioblastoma SF188 cells while Merrill et al. (2002) found that etomoxir induced oxidative stress, reduced mitochondrial membrane potential and ATP levels and increased superoxide generation. Assessing these results, it is likely that treatment with etomoxir has a cumulative effect, whereby the inhibition of fatty acid metabolism may play a role in the reduction in fusion rates and this is exacerbated by the toxic effects of etomoxir, such as the decrease in mitochondrial membrane potential which has already been shown to decrease the likelihood of a fusion event occurring (Gilad et al., 2008).

Pyruvate constitutes a critical branch point in cellular carbon metabolism as it is the end product of glycolysis and a major substrate for the TCA cycle while also being involved in anabolic pathways for lipid synthesis, amino acid biosynthesis and gluconeogenesis. Transport of pyruvate into the mitochondria is performed by the MPC which has been studied extensively but the genes that encode it remain unknown (Hildyard and Halestrap, 2003), apart from two recently identified mitochondrial inner membrane proteins (MPC1/2). In spite of this, a number of α -

cyanocinnamate analogs, such as UK5099, have been identified as specific and potent inhibitors of carrier activity (Halestrap, 1975). Inhibition of the MPC with UK5099 resulted in a significant inhibition of mitochondrial fusion in differentiated PC12 cells (Fig 4.5). Interestingly, there was only about a 30% inhibition of fusion observed after UK5099 treatment, which is less than one would expect by completely blocking pyruvate transport into the mitochondrion as it is the main substrate of the TCA cycle. One reason for the low rate of fusion inhibition observed is possibly due to the passive diffusion of pyruvate across the mitochondrial membrane that is not inhibited by UK5099 and provides another means of entry into the TCA cycle (Hildyard et al., 2005). Most interestingly, co-incubation with UK5099 and etomoxir resulted in cumulative effect of fusion inhibition with around 60% overall inhibition (Fig 4.6). This result suggests that β -oxidation of fatty acids may have a more significant input into neuronal metabolism than is currently appreciated and certainly plays a role in the maintenance of mitochondrial fission/fusion dynamics. It would be interesting to test the effect of these inhibitors on respiration using the seahorse extracellular flux analyser to see any decreases observed in oxygen consumption rates or if the deficits in fusion may be caused by toxic insults and initiation of cleavage of Opa1 in this manner.

Phenylpyruvate has been shown to competitively inhibit acetyl-CoA carboxylase in extracts from rat brains (Land and Clark, 1973). Acetyl CoA carboxylase catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA through its two catalytic activities, biotin carboxylase (BC) and carboxyltransferase (CT) (Vagelos et al., 1963). By incubating PC12 cells with phenylpyruvate, we expected a decrease in malonyl CoA levels and a subsequent increase in flux through the fatty acid β -oxidation pathway, which may have led to an increase in mitochondrial fusion. However, a significant decrease was observed (Fig 4.7). This result was not entirely surprising as phenylpyruvate has a wide range of substrates involved in a variety of metabolic pathways, which may have impacted ATP production and/or the ability of the mitochondria to fuse. Phenylketonuria is an inherited disorder resulting in decreased metabolism of the amino acid phenylalanine as a result of a mutation in the gene for the hepatic enzyme phenylalanine hydroxylase (PAH),

rendering it non-functional. The resultant accumulation of phenylalanine is converted into phenylpyruvate (Christ, 2003). Phenylketonuria presents with symptoms affecting the brain including microcephaly, hyper-activity and severe learning disabilities (Hafid and Christodoulou, 2015). It is important to note that excess of phenylpyruvate here disrupts mitochondrial dynamics and this may be responsible for some of the symptoms seen in phenylketonuria along with the described reduction in myelination (Land and Clark, 1973). A more specific inhibitor of acetyl CoA carboxylase would provide more conclusive evidence on the impact of fatty acid oxidation on mitochondrial fusion in differentiated PC12 cells.

The mitochondrial pyruvate dehydrogenase complex (PDC), of which PDH is the first enzyme, catalyses the oxidative decarboxylation of pyruvate to acetyl CoA to meet the requirements of the TCA cycle. When glucose supply is in excess, the combination of mitochondrial acetyl-CoA with oxaloacetate, via citrate formation and efflux, provides a precursor for malonyl CoA production and so limits the oxidation of fatty acids. PDH is subject to a continuous phosphorylation (inactivation) – dephosphorylation (activation) cycle catalysed by PDK, dedicated regulatory enzymes which phosphorylate and inactivate PDH, and pyruvate dehydrogenase phosphatases (PDPs) which dephosphorylate and activate PDH (Holness and Sugden, 2003). DCA is an inhibitor of PDK, and thus activates PDH promoting the conversion of pyruvate to acetyl-CoA and thus encouraging glucose use as a primary energy source. As seen in Fig 4.8, treatment with 10 mM DCA has no effect on mitochondrial fusion, suggesting that pyruvate utilization is already sufficient to drive normal mitochondrial fusion. Indeed, considering other results shown in this study, it is likely that this is the optimum or maximal rate of fusion in differentiated PC12 cells. Due to some toxic side effects of etomoxir, it wasn't clear whether the decrease in fusion rates observed with 100 μ M treatment were due to a decrease in fatty acid oxidation or possible oxidative stress. Treatment with DCA overcomes the decrease in fusion rates seen with 100 μ M etomoxir treatment and returns fusion rates to control levels. This result suggests that the inhibition of fusion seen after etomoxir treatment was due to a decrease in fatty acid oxidation and subsequent reduction in mitochondrial acetyl CoA levels but that increasing

pyruvate decarboxylation can compensate for any loss of fatty acid oxidation. Many patients with defects in long chain fatty acid oxidation present with neurological symptoms (Kompore and Rizzo, 2008) and altered lipid metabolism has also been identified in patients with a number of neurodegenerative disorders (Adibhatla and Hatcher, 2008). These results suggest that DCA could be a potential therapeutic for patients with defective lipid metabolism by increasing flux through pyruvate and stimulating a maintenance of mitochondrial fusion and general cellular homeostasis.

ACAD9 is a gene that bears close homology to VLCAD first discovered by Zhang et al. (2002) which has been shown to have dual roles in fatty acid oxidation and complex I assembly (Nouws et al., 2014, Scheffler, 2010). Previous data from the lab has shown ACAD9 knockdown decreases cell growth and survival in murine embryonic stem (ES) cells and induced pluripotent stem (iPS) cells as well as increasing ROS production and decreasing the neurogenic and neuroitogenic capacity of these cells. Expression of ACAD9 and complex I subunit NDUFA9 are both decreased in differentiated PC12 cells when compared to differentiated PC12 cells (Fig 4.10). This is surprising as differentiated cells are larger and rely more on OXPHOS for ATP production and so one would expect a higher level of complex I gene expression. In the case of ACAD9 it may be expected, as increased complex I has already been assembled when cells are full differentiated. The decrease in the NDUFA9 subunit may be explained by an increase in supercomplex formation in the differentiated cells, giving more efficient OXPHOS and reducing the need for high levels of complex I protein expression. Further work is needed to investigate this phenomenon.

No differences were noticed in cell viability or neurite outgrowth in the ACAD9 knockdown cells. Additionally, as seen in Fig 4.14, 4.15 and 4.16, no significant differences were observed in mitochondrial fusion, $\Delta\psi_m$ or ROS production in these cells. $\Delta\psi_m$ is slightly reduced which may indicate a decrease in complex I activity but this is not sufficient to affect mitochondrial fusion indicating the cells must compensate for this. It would be interesting to carry out complex I activity assays and seahorse experiments to assess the effect of the knockdown on cellular

bioenergetics. Perhaps the most likely explanation for the lack of changes in phenotype observed is the relatively low expression of ACAD9 in differentiated PC12 cells, indicating that its knockdown can be compensated by other complex I assembly factors and it may not have any catalytic activity in fatty acid oxidation. Preliminary experiments have shown that expression is higher in primary cortical neurons so it will be interesting to investigate the effect of a knockdown in these cells.

Overall, these results show that regulating metabolic flux in the mitochondrion can affect mitochondrial fusion in differentiated PC12 cells. Importantly, fatty acid oxidation may play a larger role in maintenance of mitochondrial fusion in this cell type than previously anticipated, although this effect is not mediated through ACAD9 in PC12 cells.

Chapter 5

Investigating the Effect of O-
GlcNAcylation on Mitochondrial
Function in Differentiated PC12 Cells

5.1 Introduction

O-GlcNAcylation is emerging as an important mechanism for the regulation of a wide variety of physiological processes in eukaryotic cells, particularly linking metabolism and mitochondrial function. To date, over 1000 protein targets of O-GlcNAc have been identified which have functions involving the regulation of transcription, epigenetics and cell signaling. Additionally, 88 mitochondrial glycoproteins have been identified, including 4 encoded by mtDNA highlighting the possibility that proteins can be O-GlcNAcylated within the mitochondria. This chapter will examine the effect of O-GlcNAc cycling on mitochondrial function within the parkinsonian PC12 neuronal model system.

5.1.1 O-GlcNAc Cycling and Mitochondrial Function

As a result of the well-established link between diabetes and cardiovascular disease, much of the research on O-GlcNAcylation and mitochondria has been carried out in cardiac myocytes, where altering O-GlcNAc levels has been shown to have a variety of bioenergetic and dynamic effects. Hu et al. (2009) found that high glucose treatment increased O-GlcNAcylation levels of mitochondrial proteins in cardiac myocytes and this reduced the activity of complex I, III and IV as well as reducing mitochondrial ATP and calcium content. Other studies have shown that decreasing O-GlcNAcylation through siRNA mediated knockdown of OGT increases mitochondrial respiration and glycolysis in cancer cells and that the mitochondrial form of OGT (mOGT) is involved in the glycosylation of a restricted set of mitochondrial protein targets including glycerol-3-phosphate dehydrogenase and leucine-rich PPR motif-containing protein (Sacoman et al., 2017). Overexpression of OGA or OGT in a neuronal cell line (SY5Y cells) resulted in a significant decrease in mitochondria localized proteins and a decrease in both respiration and glycolysis (Tan et al., 2014). The variance in these results clearly shows that O-GlcNAc cycling has an important role in mitochondrial function through post-translational modification of individual components of the bioenergetics system. Correct function of this system clearly requires a balance of

addition/removal of O-GlcNAc on target proteins and subtle defects in this balance could severely affect cellular function over long term periods.

However, other groups have focused on the overall effect O-GlcNAcylation has on metabolism and metabolic signaling, for example insulin signaling. After insulin binding to the insulin receptor, OGT localizes to the plasma membrane, O-GlcNAcylation of insulin receptor substrate 1 (IRS1), resulting in a suppression of insulin signaling (Whelan et al., 2008, Whelan et al., 2010b). An increase in glucose levels also increases GlcNAcylation of glycogen synthase and CRTC2 (cyclic adenosine monophosphate response element binding protein 2), decreasing glycogen synthase function and increasing gluconeogenesis. Concomitantly, increased GlcNAcylation of FOXO1 increases the transcription of a variety of gluconeogenesis genes including glucose-6-phosphatase (Housley et al., 2008). This example shows the effect of altered glucose uptake on metabolic signaling within the cell which can strongly influence metabolism, before taking into account the effect of O-GlcNAc cycling on individual enzyme complexes within the mitochondria, highlighting how tightly regulated this system needs to be in order to maintain regular mitochondrial function.

Calcium flux in cells has also been found to be altered in response to varying levels of O-GlcNAcylation which could be especially important in neuronal cells for trafficking of mitochondria and neurotransmitter release. Increased O-GlcNAcylation in response to high glucose exposure impairs calcium flux in cardiac myocytes, through altered activity of the nuclear transcription factor Sp1, reducing expression of sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA2a) (Clark et al., 2003). Additionally, depleting protein O-GlcNAcylation through overexpression of OGA has been shown to improve calcium handling and contractile function in mouse hearts that also displayed a 40% increase in SERCA2a expression (Hu et al., 2005). Finally, alterations in O-GlcNAc cycling have been linked to changes in mitochondrial morphology, which links this post-translational modification to mitochondrial dynamics. Reduction of OGT has been shown to elicit mitochondrial fragmentation, while high glucose treatment, increasing O-GlcNAc levels has also

been shown to elicit this effect. This suggests that the level of O-GlcNAc cycling is important in determining morphology in a particular cell type (Makino et al., 2011, Sacoman et al., 2017). Both Drp1 and Opa1 have been shown to be O-GlcNAcylated; increased O-GlcNAcylation of Drp1 promotes translocation to mitochondria and augments levels of the GTP-bound active form (Gawlowski et al., 2012), and increased cellular O-GlcNAcylation has been shown to reduce Opa1 protein expression and activity (Makino et al., 2011). Again, the somewhat contradictory effects seen with high glucose treatment and OGA/OGT knockdown suggest that this may be a cyclical effect which varies between cell types, highlighting the need for further investigation into a neuronal phenotype. This may prove to have important considerations in the future treatment of neurodegenerative diseases, which have already been linked to altered glucose metabolism and dysfunctional O-GlcNAc cycling.

5.1.2 O-GlcNAc and its Implications in Neurodegenerative Diseases

The finding that O-GlcNAc is present on proteins such as tau and α -synuclein, along with the knowledge that O-GlcNAc levels altered with aging, have increased research into the possibility that aberrant O-GlcNAcylation may be involved in the progression of neurodegenerative diseases, particularly AD and PD.

In AD, expression of GLUT1 and GLUT3 glucose transporters is decreased on the cell surface, which is unsurprising considering a common hallmark of neurodegeneration is decreased glucose uptake. However, the number of insulin receptors, which would usually stimulate GLUT3 expression, is decreased, hinting that a defect in insulin signaling may be a risk factor for AD development, as in type 2 diabetes (Zhu et al., 2014). Indeed, treatments that enhance insulin signaling have been shown to decrease A β and tau plaque formation as well as blocking some of the toxic effects on synaptic function and long-term potentiation (Townsend et al., 2007). The negative effects of this dysregulation of insulin signaling may be connected to altered O-GlcNAc cycling as both pharmacological and genetic mechanisms to enhance O-GlcNAcylation have been reported to have

neuroprotective properties in AD models. Several groups have reported that long term treatment with specific inhibitors of OGA decreases the number of degenerative lesions in the brain, and reduces toxic A β levels and plaque formation (Graham et al., 2014, Yuzwa et al., 2014). Amyloid precursor protein, γ -secretase and tau are O-GlcNAcylated and it is hypothesized that increased levels of O-GlcNAc may stimulate production of the non-toxic form of A β and decrease phosphorylation of tau, leading to a decrease in aggregation.

As stated above, the link between PD and altered O-GlcNAcylation is also being investigated. The main hallmark of PD is α -synuclein deposits in the brain and O-GlcNAc sites have been found on threonine 53, 64 and 72 of the NAC region in rat brain, which is required for aggregation (Alfaro et al., 2012). These O-GlcNAc sites are located in a region prone to self-aggregation, so it is possible that this modification may affect the aggregation of α -synuclein (Murray et al., 2003). Studies have begun to emerge that show increased levels of O-GlcNAcylation decreases α -synuclein toxicity when added exogenously to cells in culture (Marotta et al., 2015), strengthening the need for further research into this area. In addition to these preliminary results, PD brains have also been shown to have decreased glucose metabolism in cortical and subcortical regions of the brain. Combined, these data suggest that altered O-GlcNAc levels may play a role in aging and PD development. The initial insult may be a defect in glucose metabolism, which decreases global O-GlcNAc levels, increasing α -synuclein aggregation, and subsequently affecting mitochondrial/cellular function but more work is needed to elucidate the full function of O-GlcNAc cycling in the brain.

5.1.3 Aims of the Chapter

- To alter O-GlcNAc levels in differentiated PC12 cells by pharmacological and genetic mechanisms.
- To quantify the effects of altered O-GlcNAc levels on mitochondrial fusion in differentiated PC12 cells.
- To investigate any changes in $\Delta\psi_m$, ROS production, mitochondrial morphology and bioenergetics in differentiated PC12 cells with altered O-GlcNAc levels.
- To characterize the effects of altered O-GlcNAc levels on electron transport chain activities in differentiated PC12 cells.

5.2 Methods

5.2.1 Measurement of O-GlcNAc Levels

Differentiated PC12 cells were treated with the OGA inhibitor, thiamet G, for 16 hours or the OGT inhibitor, ST04589 (TT04) for 4 hours at 37 °C before being lysed using RIPA buffer as described previously (section 2.11). Cells stably expressing siRNA for OGA or OGT (or non-target control) were lysed 48 hours after differentiation in a similar way. Samples were then sonicated and processed through gel electrophoresis and western blot to visualize levels of O-GlcNAc in the various cell lines.

5.2.2 Generation of Stable PC12 Cells Expressing OGA/OGT shRNA

PC12 cells were seeded in a 96 well plate at a density of 2.0×10^4 cells/well in fresh media and were incubated overnight in a humidified incubator at 37 °C, 5% CO₂. The following day, media was changed and based on the optimized multiplicity of infection (MOI) of 20 for PC12 cells, lentiviral particles (3 constructs for each gene) were added to each well and incubated at 37 °C overnight. After 24 hours, media containing lentiviral particles was removed and cells were maintained in normal RPMI 1640 (10% FBS, 1% P/S), the following day, media was replaced with fresh media containing 800ng/ml puromycin to begin the selection process. After 3-4 days, 3 resistant colonies for each construct were selected and expanded to be assayed for total O-GlcNAc levels by western blot. At this point, OGA knockdown cells which showed the greatest increase in global O-GlcNAc levels, as well as OGT knockdown cells which showed the greatest decrease in global O-GlcNAc levels, were used for future experiments. The constructs were designed by Sigma Aldrich using the Rosetta algorithm and siRNA sequences were as follows.

O-GlcNAcase-1 GTGGGATATCAAGAGTATAAT

O-GlcNAcase-2 GAGTGCTGACTAATCCAAATT

O-GlcNAcase-3 AGGAGGTATTACCAGAACTT

O-GlcNAc transferase-1 GAGCCCAATATTCAACAATAT

O-GlcNAc transferase-2 CTGCTGCACATTCCAATTTAG

O-GlcNAc transferase-3 CGGCTCAAGCCTGATTTCATT

Non-Target Control – Sigma Aldrich

5.2.3 Cell Viability

PC12 (6-15) or PC12 OGA/OGT KD cells were seeded in 96 well plates coated with poly-D-lysine and differentiated with nerve growth factor (NGF) for 48 hours. Control cells were treated with thiamet G (100 nM) or TT04 (2 μ M) for 4 hours. AlamarBlue[®] solution was added (1:10) to each well and plates were incubated for 4 hours before fluorescence (excitation λ =570 and emission λ =600) were measured.

5.2.4 Mitochondrial Morphology

Cells were transfected with mDS-Red7 as described in section 2.8. Briefly, PC12 (6-15) SCR/OGA/OGT cells were seeded in 6 well plates at a density of 200,000/well and incubated at 37 °C overnight. The following day the cells were incubated with 6 μ l Lipofectamine 2000[®] and 2 μ g mDSRed7 plasmid in 500 μ l OptiMEM and 2000 μ l antibiotic free RPMI 1640 for 4 hours before being replaced with fresh fully supplemented media. The following day, media was removed, cells were trypsinized and transferred to a 24 well plate containing 13 mm glass coverslips, coated with poly-D-lysine, at a density of 100,000 cells/well in RPMI 1640 (1% FBS, 1% P/S) and differentiated with NGF. After 48 hours, media was removed, cells were washed 3 times with PBS and fixed using 4% PFA for 15 mins. Cells were then mounted using hydromount (National Diagnostics, USA) and sealed using nail varnish. Cells were then imaged using a Leica Sp8 confocal microscope and de-convoluted using Hyugens max software. At least 10 fields of view were taken for each slide on three separate occasions giving a minimum of 30 cells of which representative images are shown.

5.2.5 Measurement of Respiratory Chain Complex Activities

Differentiated PC12 SCR/OGA/OGT cells were washed, scraped and spun down before homogenized and freeze fractured three times in liquid nitrogen. The activities of complex I, II/III and IV were then measured. Experiments were carried out at 37 °C in a Shimadzu spectrophotometer.

To measure complex I activity, 50 µg protein was added to a cuvette containing assay buffer (5 mM KH₂PO₄, 10 mM MgCl₂, pH 7.4), 0.2 mM NADH, 2.5 mg BSA and 1 mM KCN, and initial rates of absorbance change were measured for 2-3 min at $\lambda=340$. First, decylubiquinone (50 µM) was added and the rates were measured for 3-4 min, followed by rotenone (10 µM) for 5 min, to obtain rotenone sensitive rates.

For complex II/III activity, 10 µg protein was added to cuvettes containing assay buffer (100 mM KH₂PO₄, 0.3 mM K-EDTA, pH 7.4), 20 mM succinate, 1 mM KCN and rates were measured at $\lambda=550$. The initial rates were measured for 2-3 min before cytochrome *c* (100 µM) was added to start the reaction and the rates were measured for 6-9 min. Antimycin A (1 µM) was then added, and the rates were recorded for another 5 min.

To measure complex IV activity, cytochrome *c* was reduced by the addition of ascorbic acid to oxidized cytochrome *c* (25 mg/ml), which was removed by filtration through a PD₁₀ column. The column had been pre-rinsed with potassium phosphate buffer (10 mM, pH 7.4). 50 µg protein was then added to a cuvette containing assay buffer (100 mM KH₂PO₄, pH 7.4) and absorbance was measured at $\lambda=550$ for 2 min before cytochrome *c* (50 µM) was added and rates were followed for another 10 min.

5.2.6 Citrate Synthase Activity Assay

Citrate synthase is an enzyme in the citric acid cycle. It is localized in the mitochondrial matrix and is often used as a quantitative marker enzyme for the

content of mitochondria within cell or tissue samples. Citrate synthase enzyme activity was measured spectrophotometrically by a colored coupled reaction using a method adapted from that originally described by (Srere, 1969). The activity of citrate synthase was determined by monitoring the rate of production of thionitrobenzoic acid (TNB) at a wavelength of 412 nm. PC12 cell homogenate (50 μ g) was incubated in a 1 ml cuvette with tris buffer (200 mM, pH 8.1) and the following reaction components were added; 5,5' dithiobis-(2-nitrobenzoic acid (DNTB) (100 μ M), acetyl coenzyme A (300 μ M) and Triton X-100 (0.1%). A blank rate was measured for 2 min before oxaloacetate (500 μ M) was added to initiate the reaction and an increase in absorbance was measured for 3 min.

5.3 Results

5.3.1 Investigating O-GlcNAc Expression in PC12 cells treated with the O-GlcNAcase Inhibitor Thiamet G and the O-GlcNAc Transferase Inhibitor TT04

Figure 5.1 shows O-GlcNAc levels in PC12 cells incubated with 100 nM thiamet G for 16 hours and 2 μ M TT04 for 4 hours causing alterations in global cellular O-GlcNAc levels. These results show that O-GlcNAcylation is active in differentiated PC12 cells and can be altered by the inhibition of the enzymes responsible for the addition and removal of the sugar.

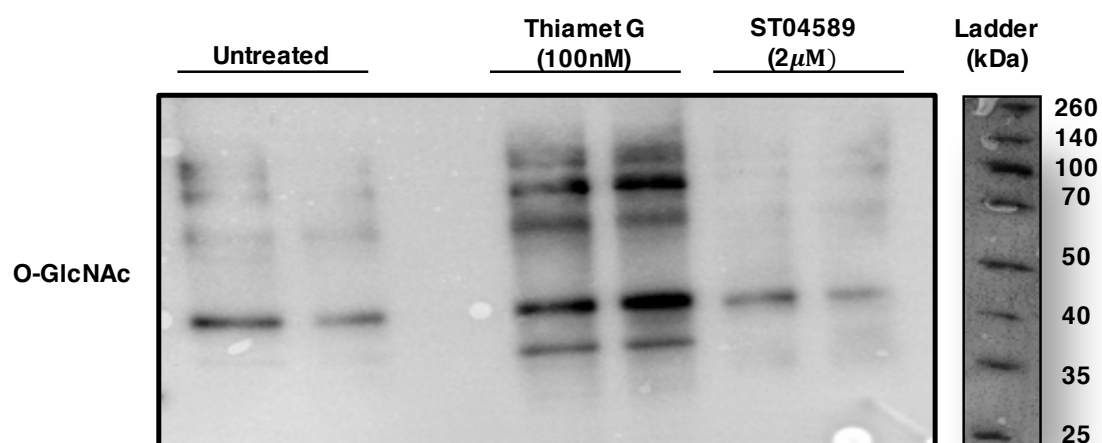


Figure 5.1: Treatment with inhibitors of O-GlcNAcase or O-GlcNAc transferase can alter O-GlcNAc levels in differentiated PC12 cells. PC12 cells were incubated with 100 nM thiamet G at 37 °C for 16 hours or 2 µM ST045849 (TT04) for 4 hours before being washed with PBS and lysed using RIPA buffer. Lysates (20 µg) were resolved by gel electrophoresis on 12% SDS-polyacrylamide gels, followed by transfer onto a polyvinylidene difluoride (PVDF) membrane. The membranes were probed with an anti-O-linked N-acetylglucosamine antibody and visualized using a Fuji X-ray film processor RG II. Alterations in global O-GlcNAc levels can be seen from whole cell lysates. Image representative of n=3.

5.3.2 Effect of Treatment with Thiamet G and TT04 on Cell Viability in Differentiated PC12 cells

Differentiated PC12 cells treated with 100 nM thiamet G for 16 hours have no decrease in cell viability as compared to untreated control cells. In contrast, treatment with 2 μ M TT04 for 4 hours shows a 20% reduction in cell viability as seen in Fig 5.2. This suggests that an overall decrease in O-GlcNAcylation causes some cell death over this time period. This may be due to toxic effects of the drug itself or that the decrease in O-GlcNAc levels causes an increase in apoptosis.

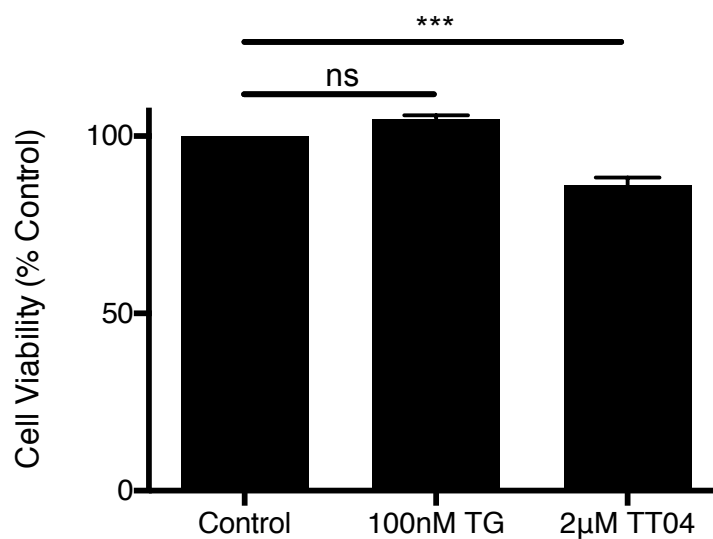


Figure 5.2: Treatment with TT04 decreases cell viability in differentiated PC12 cells. Differentiated PC12 cells were treated with 100 nM thiamet G (TG) for 16 hours or 2 μ M TT04 for 4 hours and cell viability was measured by fluorescence after the cells were incubated with alamarBlue [®] (10%) for 4 hours (excitation λ =570 and emission λ =600). Data is presented as percent of control $\pm$ SEM; n=3. A one-way ANOVA followed by Dunnett's multiple comparisons test was performed and values which were significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$, **** for $p \leq 0.0001$.

5.3.3 Effect of Inhibition of O-GlcNAcase and O-GlcNAc Transferase on Mitochondrial Fusion in Differentiated PC12 cells

Previous studies have shown that increase of O-GlcNAcylation reduces mitochondrial motility by increasing the O-GlcNAcylation of Milton and anchoring mitochondria to areas of the cell in which glucose concentrations, and energy demands, are highest (Pekkurnaz et al., 2014). Based on these results, we hypothesized that a decrease in mitochondrial motility would also result in a decrease in mitochondrial fusion as it would limit the amount of mitochondrial contact events. To test this, differentiated PC12 cells were treated with a variety of OGA and OGT inhibitors and mitochondrial fusion was assayed.

As can be seen in Fig 5.3, pre-treatment with 100 μ M PUGNAc reduces mitochondrial fusion significantly at all time points of the experiment. 45 min after photo-activation, mean pixel intensity is reduced to ~60% of intensity after 1 min while in control cells, pixel intensity is reduced to ~37%, indicating a 36.5% reduction in mitochondrial fusion after a 6 hour incubation with PUGNAc. Treatment with 3 mM alloxan also resulted in a significant reduction in mitochondrial fusion as can be seen in Fig 5.5, however because of concerns with the specificity and toxicity of these drugs it was decided to carry out all future experiments using thiamet G to inhibit OGA. Treatment with thiamet G itself resulted in a significant reduction in mitochondrial fusion of 38% at 45 min post-activation (Fig 5.4). These results show that treatment with OGA inhibitors reduce mitochondrial fusion, possibly as a result of decreased mitochondrial motility rates.

In contrast, it would be expected that a decrease in O-GlcNAcylation as a result of treatment with OGT inhibitors may result in an increase in mitochondrial fusion rates. This could be due to the mitochondria no longer being anchored to a cellular region as a result of decreased O-GlcNAcylation of Milton. However, 4 hour pre-treatment with OGT inhibitor TT04 had no effect on mitochondrial fusion as shown in Fig 5.6

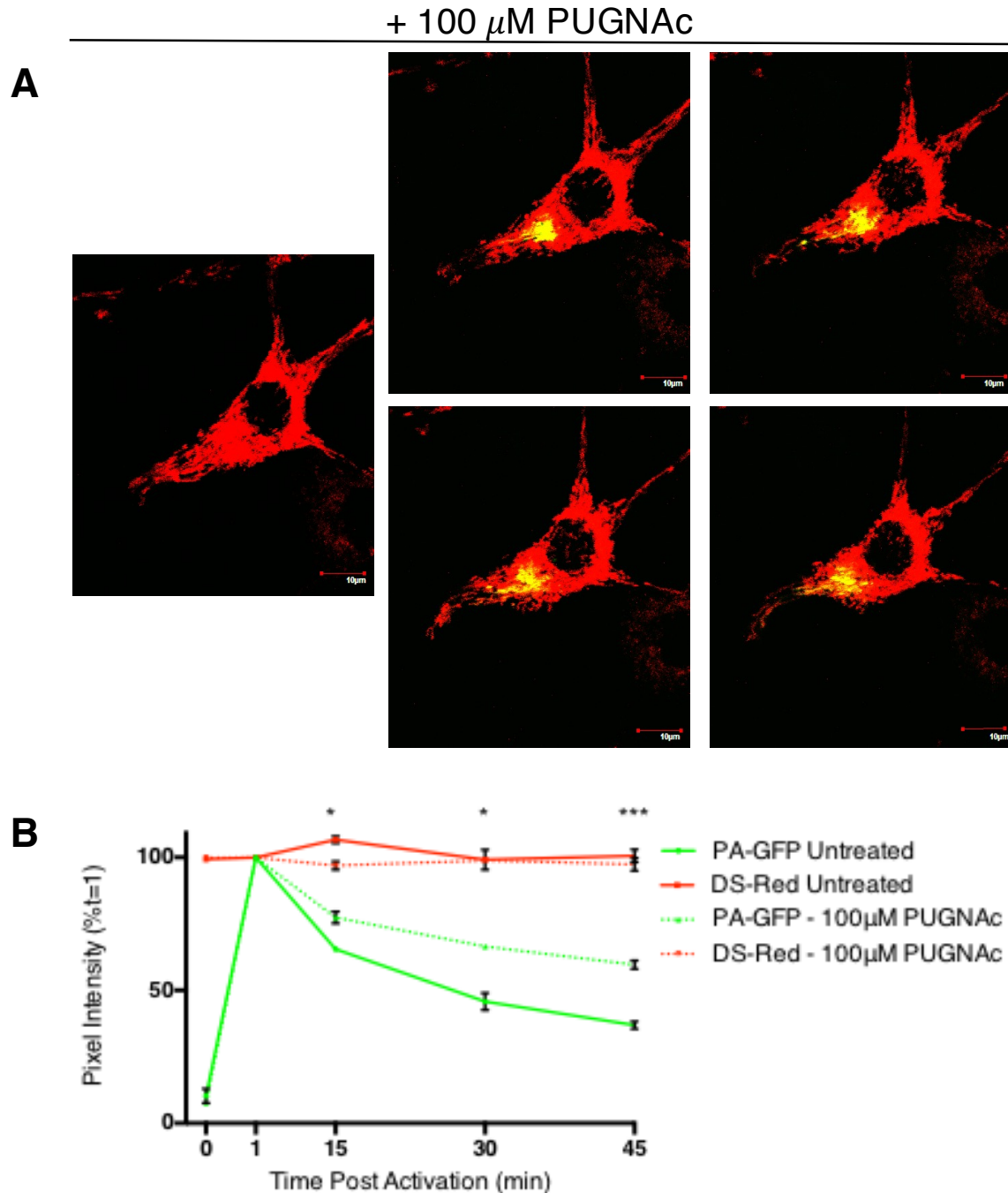


Figure 5.3: Inhibition of O-GlcNAcase with PUGNAc decreases mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 6 hour pre-incubation with 100 μ M PUGNAc on mitochondrial fusion. (B) Quantitative data shows the inhibition of fusion in PUGNAc treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, n=3. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

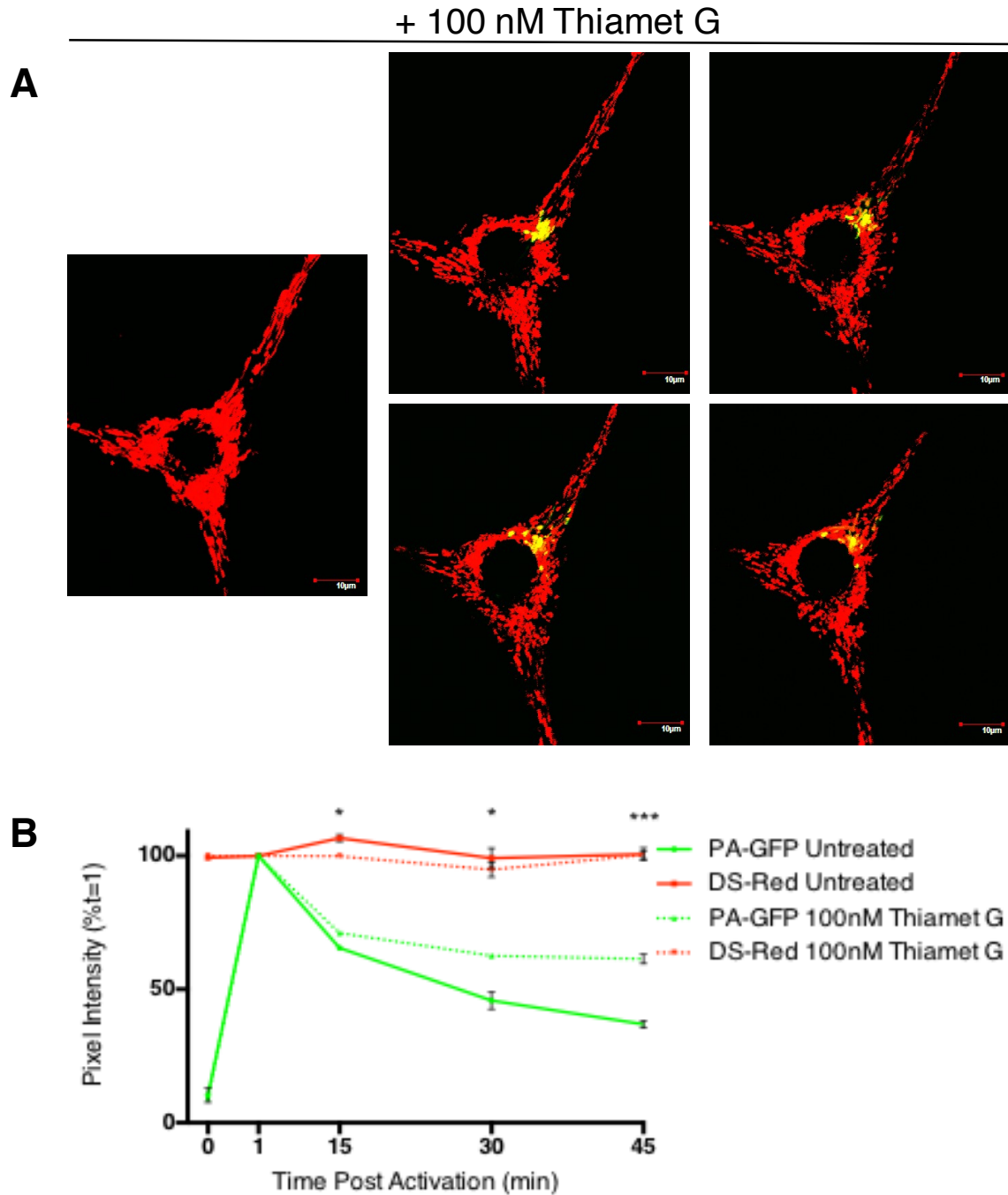


Figure 5.4: Inhibition of O-GlcNAcase with thiamet G decreases mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 16 hour pre-incubation with 100 nM thiamet G on mitochondrial fusion. (B) Quantitative data shows the inhibition of fusion in thiamet G treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

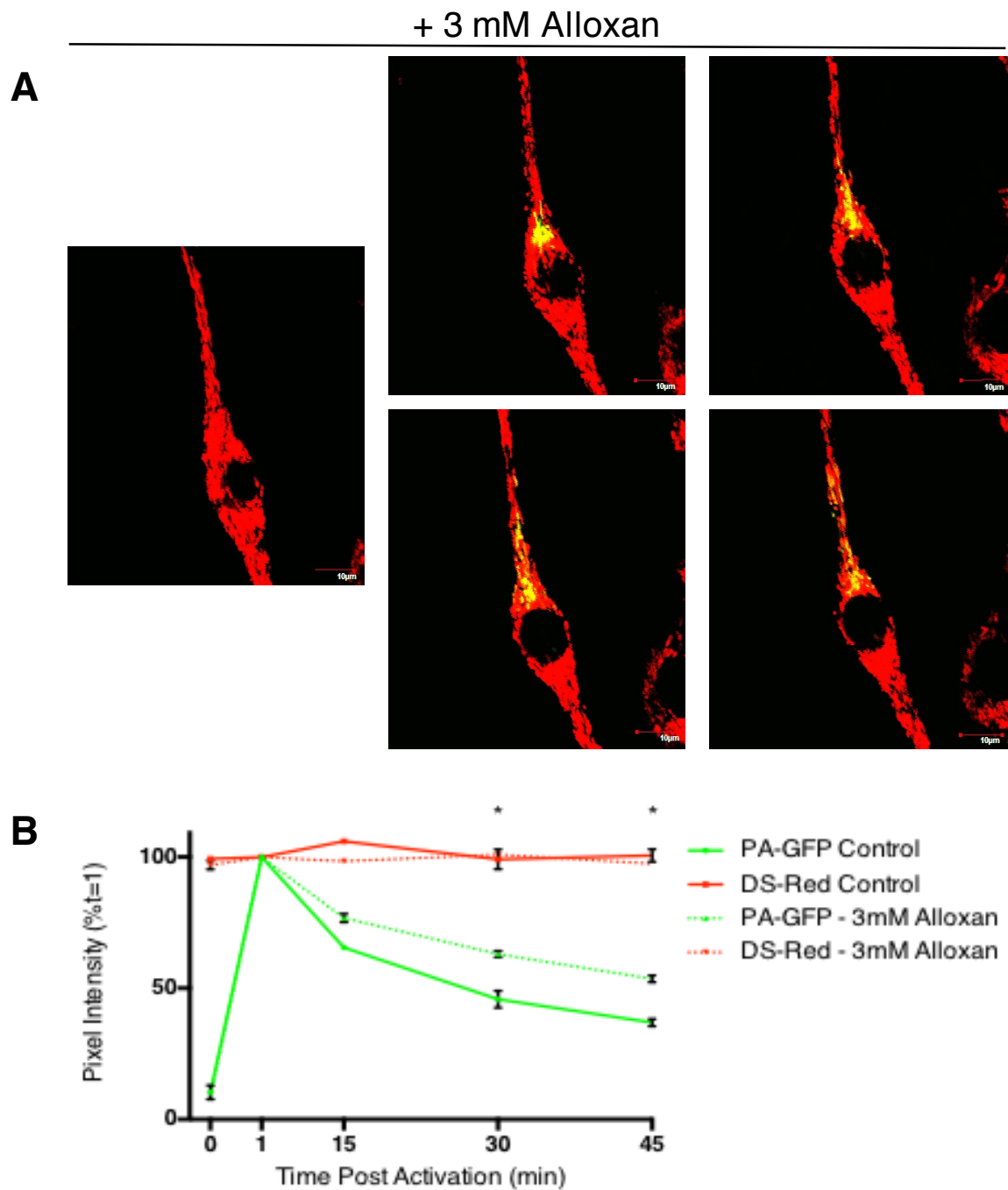


Figure 5.5: Inhibition of O-GlcNAcase with alloxan decreases mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 3 hour pre-incubation with 3 mM alloxan on mitochondrial fusion. (B) Quantitative data shows the inhibition of fusion in alloxan treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

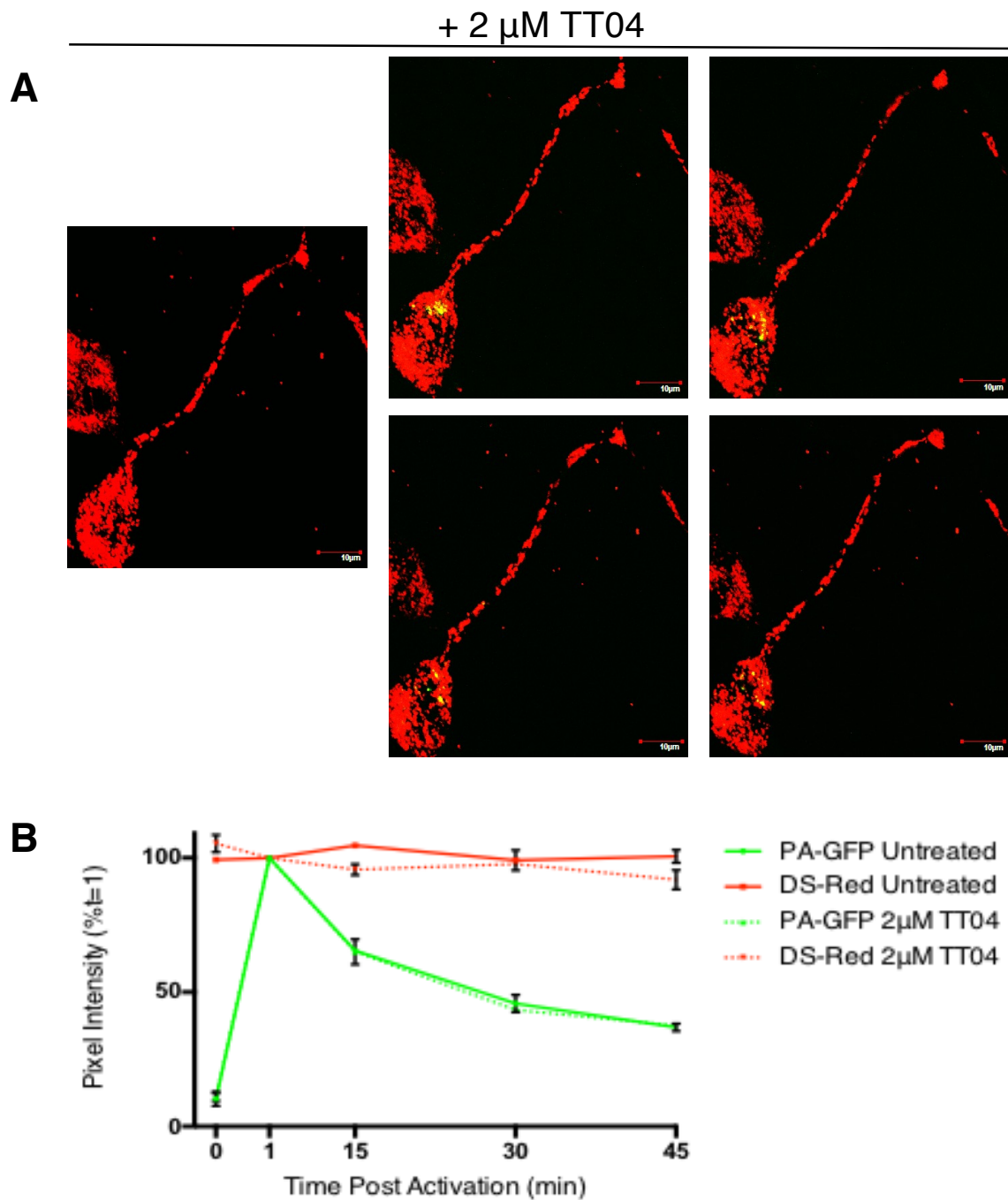


Figure 5.6: Inhibition of O-GlcNAc transferase with TT04 does not affect mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of a 4 hour pre-incubation with 2 μ M TT04 on mitochondrial fusion. (B) Quantitative data shows no inhibition of fusion in TT04 treated cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, n=3. Unpaired t-test at each time point with Welch correction for unequal variance. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

5.3.4 Effect of Inhibition of O-GlcNAcase and O-GlcNAc Transferase on $\Delta\psi_m$ and ROS Production in Differentiated PC12 cells

Alterations in O-GlcNAc levels through inhibition of OGA and OGT had no significant effect on $\Delta\psi_m$ in differentiated PC12 cells. Cells were incubated with 100 nM thiamet G for 16 hours or 2 μ M TT04 for 4 hours and loaded with 20 nM TMRM for 20 min before being imaged. As can be seen in Fig 5.7, there was a slight, non-significant decrease in pixel intensity in cells with lower O-GlcNAc levels which is somewhat surprising considering there was no effect on mitochondrial fusion and cells with a lower $\Delta\psi_m$ are thought to fuse less frequently than those with a higher membrane potential. However, it appears likely that this decrease is still above the threshold of $\Delta\psi_m$ at which mitochondrial fusion is affected.

There was no effect on ROS production in cells treated with thiamet G or TT04 (Fig 5.8) which is contrary to previous reports which showed increasing O-GlcNAcylation decreased ROS production (Ngoh et al., 2011), while a decrease in global O-GlcNAcylation levels increased ROS production as measured by H₂DCFDA (Jeon et al., 2014). Additionally, no protective or attenuating effects were seen when thiamet G or TT04 pre-treatment was combined with inhibition of the ETC as shown in Fig 5.9. Large increases in ROS production were seen with antimycin A treatment but this was not decrease or increased by alteration in O-GlcNAc levels. Inhibition of the other complexes had no significant effect on ROS production in control or treated cells. This is also the case when initial rates of ROS production are measured (data not shown).

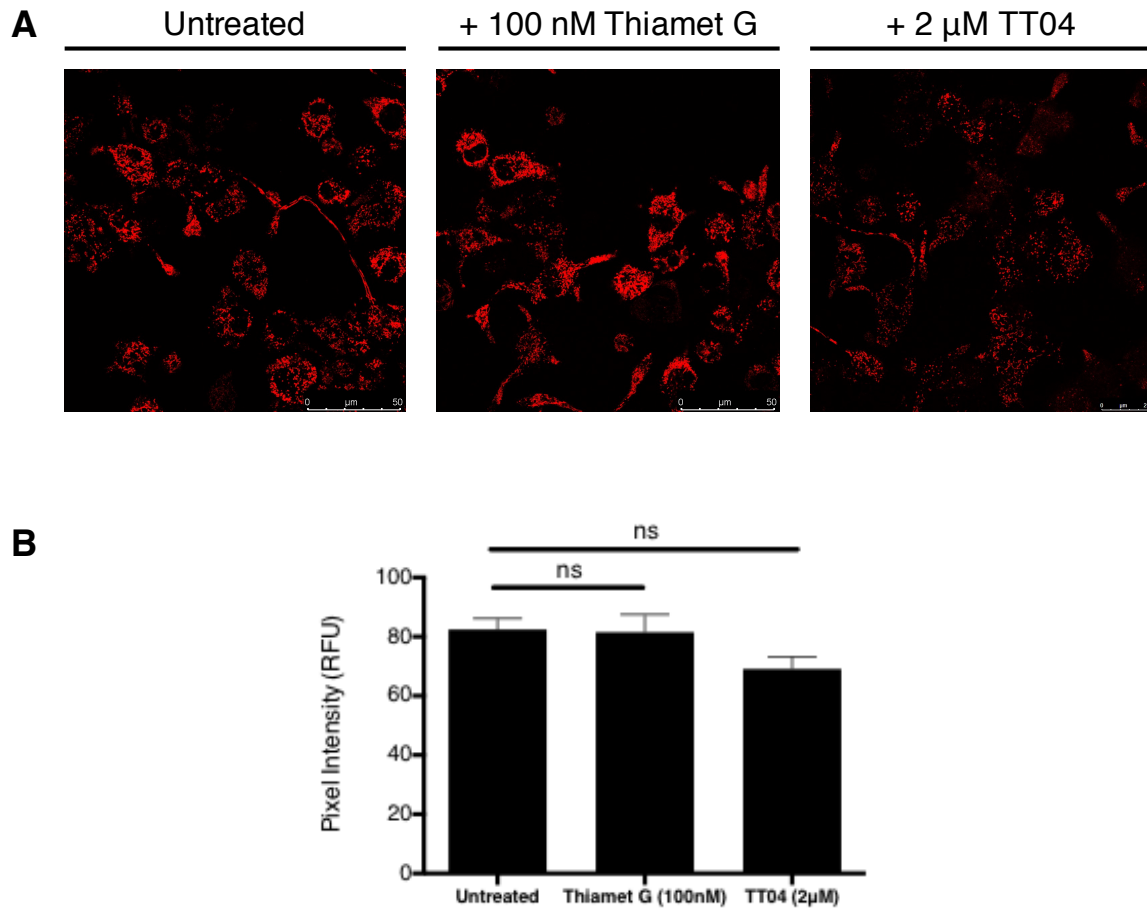


Figure 5.7: Inhibition of O-GlcNAcase or O-GlcNAc transferase has no significant effect on mitochondrial membrane potential in differentiated PC12 cells. (A) Images representative of TMRM fluorescence in cells which were untreated, treated for 16 hours with thiamet G or treated for 4 hours with TT04. (B) Analysis of pixel intensity using Imares image analysis software shows no significant difference in pixel intensity as a measure of $\Delta\psi_m$. Treatment with TT04 shows a slight non-significant reduction in pixel intensity. Data presented as mean $\pm$ SEM; n=3. A one-way ANOVA followed by Dunnett's multiple comparisons test was performed and values which are significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

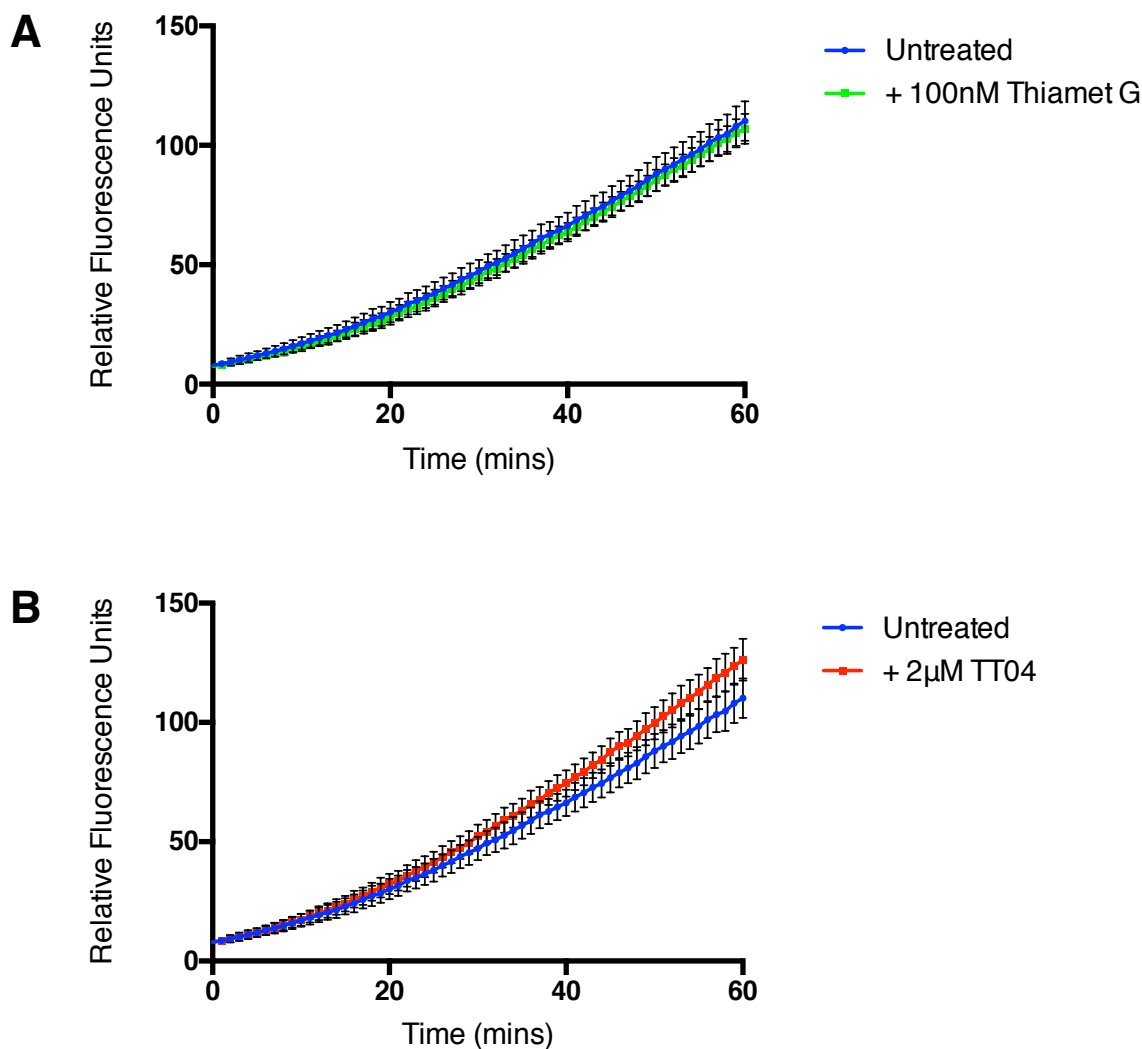


Figure 5.8: Inhibition of O-GlcNAcase or O-GlcNAc transferase has no significant effect on ROS production in differentiated PC12 cells. Differentiated PC12 cells were treated with 100 nM thiamet G (A) for 16 hours or 2 μ M TT04 (B) for 4 hours and ROS production was measured using H₂DCFDA. Cells were washed with PBS and maintained in krebs buffer containing 5 μ M H₂DCFDA while fluorescence (excitation λ =485 and emission λ =530) was measured for 1 hour. Data is expressed as relative fluorescence units $\pm$ SEM; n=3. An individual t-test was performed at each time point and values that are significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

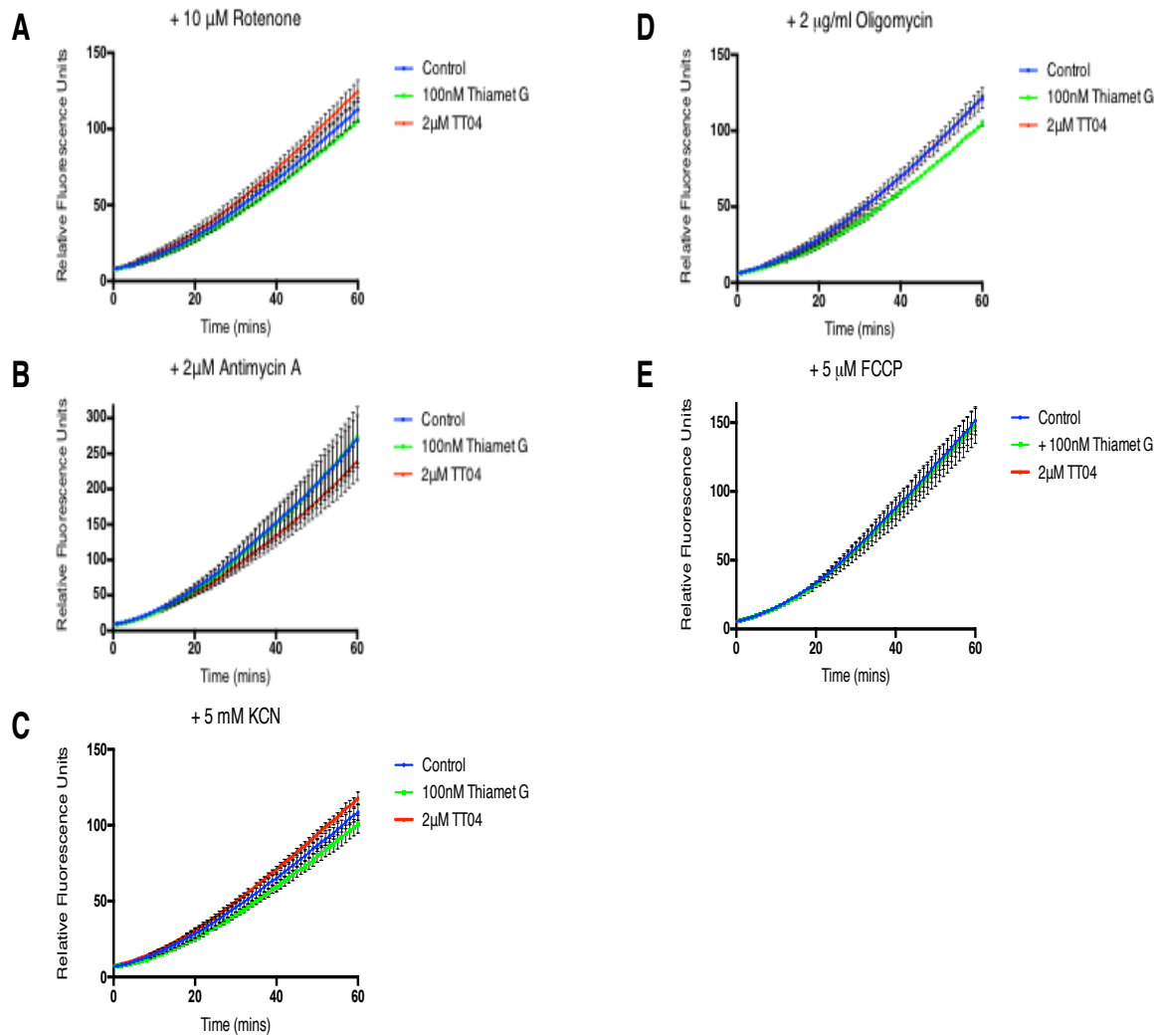


Figure 5.9: Inhibition of O-GlcNAcase or O-GlcNAc transferase in combination with OXPHOS inhibition has no significant effect on ROS production in differentiated PC12 cells.

Differentiated PC12 cells were treated with 100 nM thiamet G (A) for 16 hours or 2 μM TT04 (B) for 4 hours and ROS production was measured using H₂DCFDA in the presence of OXPHOS inhibitors. Cells were washed with PBS and maintained in krebs buffer containing 5 μM H₂DCFDA in the presence of 10 μM rotenone (A), 2 μM antimycin A (B), 5 mM KCN (C), 2 μg/ml oligomycin (D) or 5 μM FCCP (E) while fluorescence (excitation λ =485 and emission λ =530) was measured for 1 hour. Data is expressed as relative fluorescence units $\pm$ SEM; n=3. An individual t-test was performed at each time point and values that are significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

5.3.5 Knockdown of O-GlcNAcase and O-GlcNAc transferase Alters O-GlcNAc levels Without Affecting Cell Viability in Differentiated PC12 cells

Specially designed lentiviral shRNA constructs were used to knockdown the two enzymes responsible for the addition and removal of O-GlcNAc to serine or threonine residues and subsequently alter O-GlcNAc levels. PC12 cells were transduced, single colonies were chosen, selected using 800 ng/ml puromycin and expanded until enough cells could be used to lyse and carry out western blots. Initially, 3 colonies from each construct were chosen and expanded and these were analysed by western blot, the results of which allowed us to choose 3 of the expanded colonies which showed greatest alteration in O-GlcNAc levels. These were again, expanded and both differentiated and undifferentiated samples were analysed by western blot as can be seen in Fig 5.10. Densitometry analysis of these samples allowed us to choose a single expanded clone from each of the knockdowns which were termed OGA kd 2b and OGT kd 3c. These samples were expanded, frozen down and analysed by western blot to confirm alteration in O-GlcNAc levels (Fig 5.11A). Additionally, these cells were differentiated and cell viability experiments were carried out to determine if the knockdown had any effect on cell survival when differentiated. However, as shown in Fig 5.11B, cell viability was not significantly different from the scramble control.

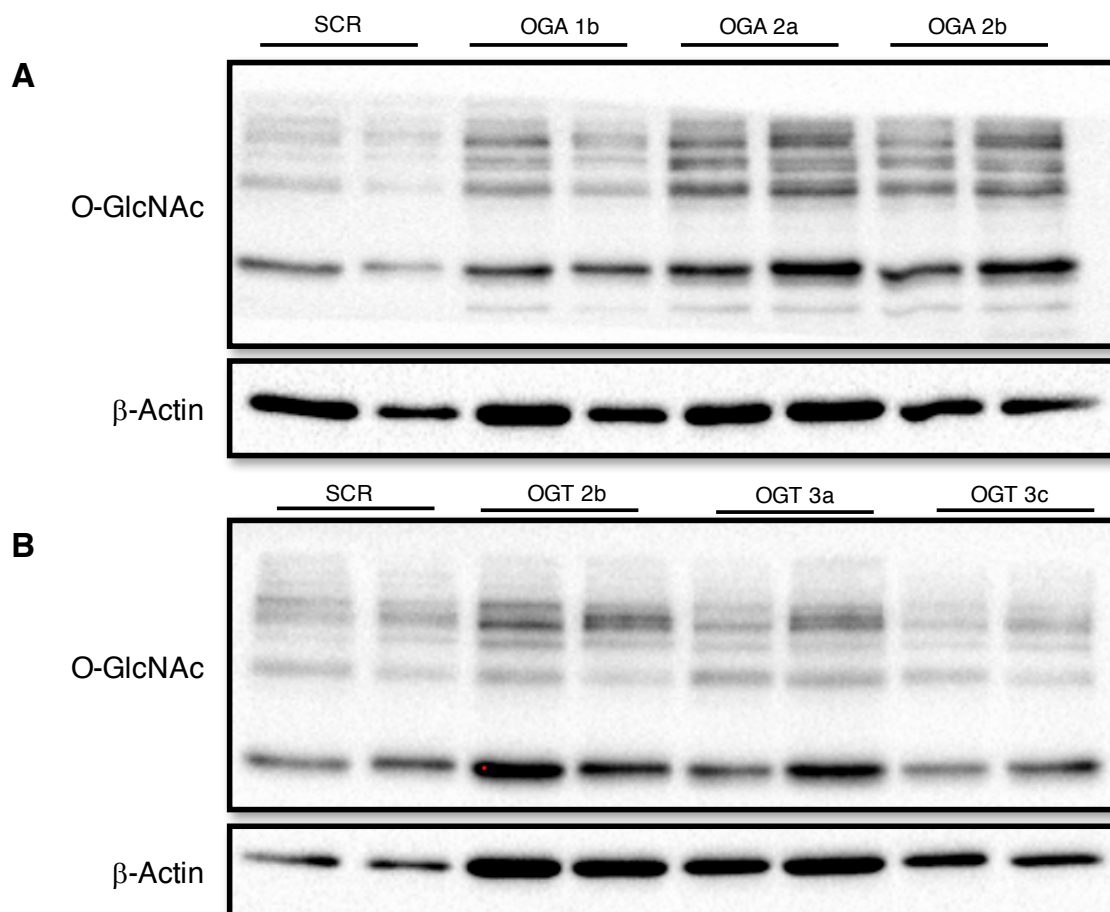


Figure 5.10: Optimization of knockdown of O-GlcNAcase and O-GlcNAc transferase. PC12 cells were transduced with lentiviral particles, targeting different regions of each gene, designed by Sigma Aldrich. Three different sequences were targeted in each gene. Cells were then selected with 800 ng/ml puromycin and expanded before being lysed and global O-GlcNAc levels were measured. Lysates were resolved by gel electrophoresis on 12% SDS-polyacrylamide gels, followed by transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were probed with an anti-O-linked N-acetylglucosamine antibody and visualized using a Fuji X-Ray film processor RG II. Alteration in O-GlcNAc expression can be seen in both OGA and OGT knockdown. Based on densitometry analysis, it was decided that constructs OGA 2b and OGT 3a provided the most efficient knockdowns.

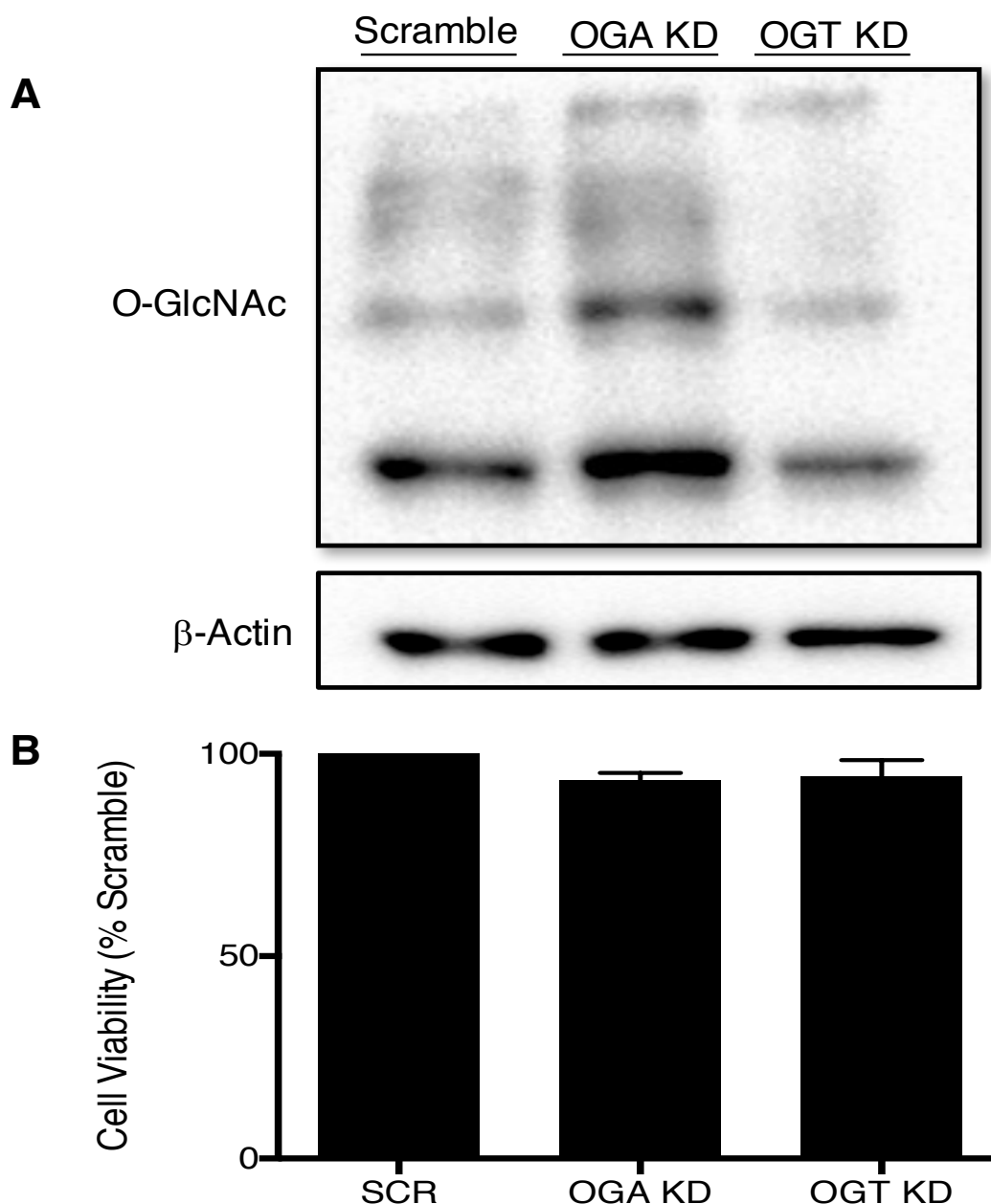


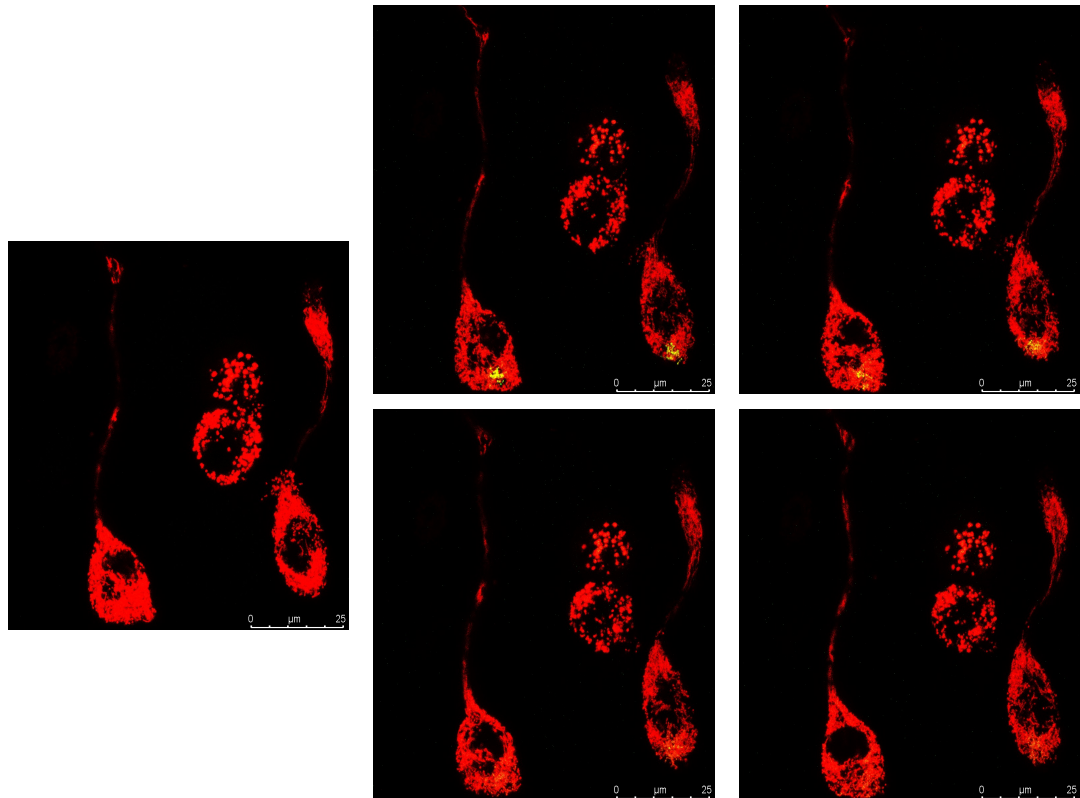
Figure 5.11: O-GlcNAcase and O-GlcNAc transferase knockdown efficiently alters total cellular O-GlcNAc levels without affecting cell viability in differentiated PC12 cells. (A) Western blot analysis of O-GlcNAc from differentiated PC12 cell lysates shows an increase in O-GlcNAc levels when OGA is knocked down and a decrease when OGT is knocked down. (B) OGT or OGA knockdown has no effect on cell viability as measured by fluorescence in cells incubated with alamarBlue® (10%) for 4 hours (excitation $\lambda=570$ and emission $\lambda=600$). Data is presented as percent of control $\pm$ SEM; $n=3$. A one-way ANOVA followed by Dunnett's multiple comparisons test was performed and values which were significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$, **** for $p \leq 0.0001$.

5.3.6 Effect of Knockdown of O-GlcNAcase and O-GlcNAc Transferase on Mitochondrial Fusion in differentiated PC12 cells

Mitochondrial fusion was measured in differentiated PC12 cells where OGA (Fig 5.12) and OGT (Fig 5.13) were knocked down. Increasing cellular O-GlcNAc levels through knockdown of OGA had no effect on mitochondrial fusion, contrary to what was seen when OGA was inhibited by thiamet G and fusion rates were significantly decreased (Fig 5.4). In contrast, no effect was seen when O-GlcNAc levels were decreased by the addition of TT04 but here we see a slight, but significant increase in mitochondrial fusion at 45 min post activation in cells where OGT is knocked down. This is interesting and may suggest that the increase in fusion rates is due to an increase in motility as a result of a decrease in Mitochondrial O-GlcNAcylation as may have been expected with TT04 treatment.

It was subsequently decided to analyse mitodynamin expression patterns (Fig 5.14) to see if any alterations could be seen. Despite the minor differences in mitochondrial fusion between knockdown and scramble cells, mitochondrial morphology is vastly different between OGA and OGT knockdown cells. As shown in Fig 5.19, scramble cells show a normal mitochondrial network with a mixture of long and short mitochondria. However, OGA knockdown cells have an excess of long, tubular mitochondria while OGT knockdown cells have rounded, punctate mitochondria and the network appears to be fragmented. While there were no changes in expression patterns of mitodynamins, this may have occurred at an earlier time point when this morphology first appeared and the cells could adapt to this phenotype over time and maintain normal fusion rates. It is also possible that alterations in O-GlcNAc levels lead to subtle changes in activity of the mitodynamins which leads to morphological changes over longer time periods.

A



B

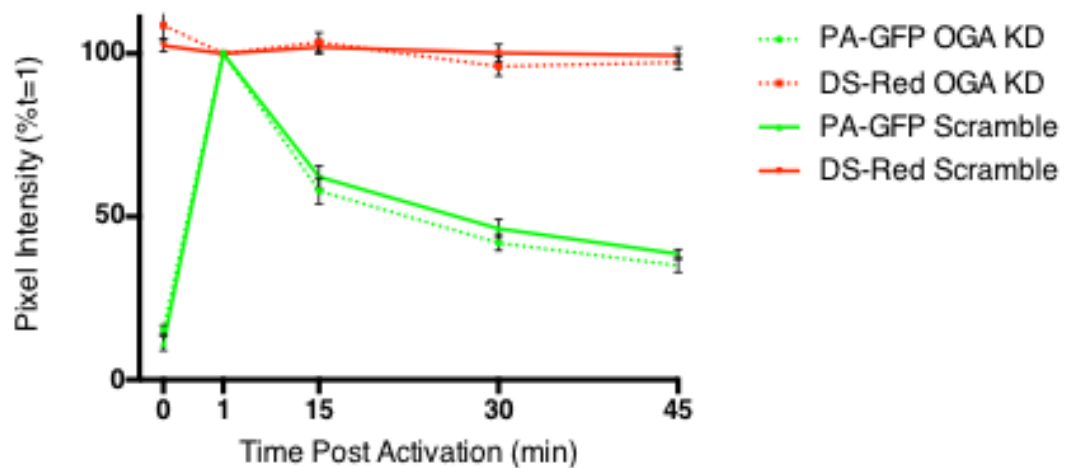


Figure 5.12: Stable knockdown of O-GlcNAcase has no effect on mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of stable knockdown of O-GlcNAcase on mitochondrial fusion in differentiated PC12 cells. (B) Quantitative data shows the rates of fusion in OGA knockdown cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. An unpaired t-test at each time point with Welch correction for unequal variance was performed. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

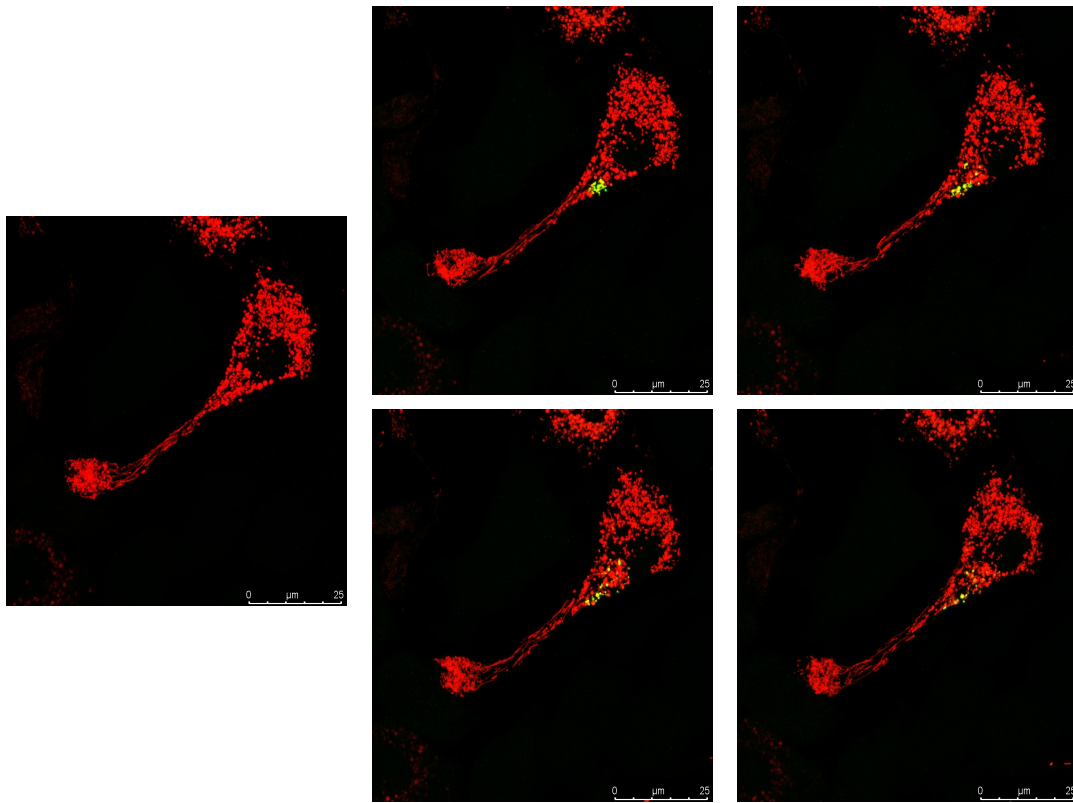
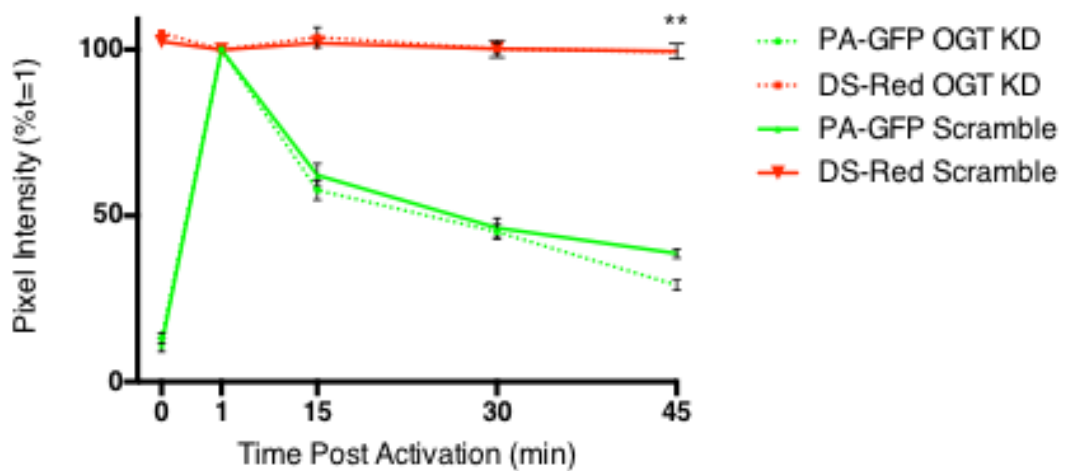
A**B**

Figure 5.13: Stable knockdown of O-GlcNAc transferase increases mitochondrial fusion in differentiated PC12 cells. (A) Images representative of the effect of stable knockdown of O-GlcNAc transferase on mitochondrial fusion in differentiated PC12 cells. (B) Quantitative data shows the rates of fusion in OGT knockdown cells (dashed green line) compared to controls (bold green line). Data presented as mean $\pm$ SEM, $n=3$. An unpaired t-test at each time point with Welch correction for unequal variance was performed. Results which were significantly different from controls are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

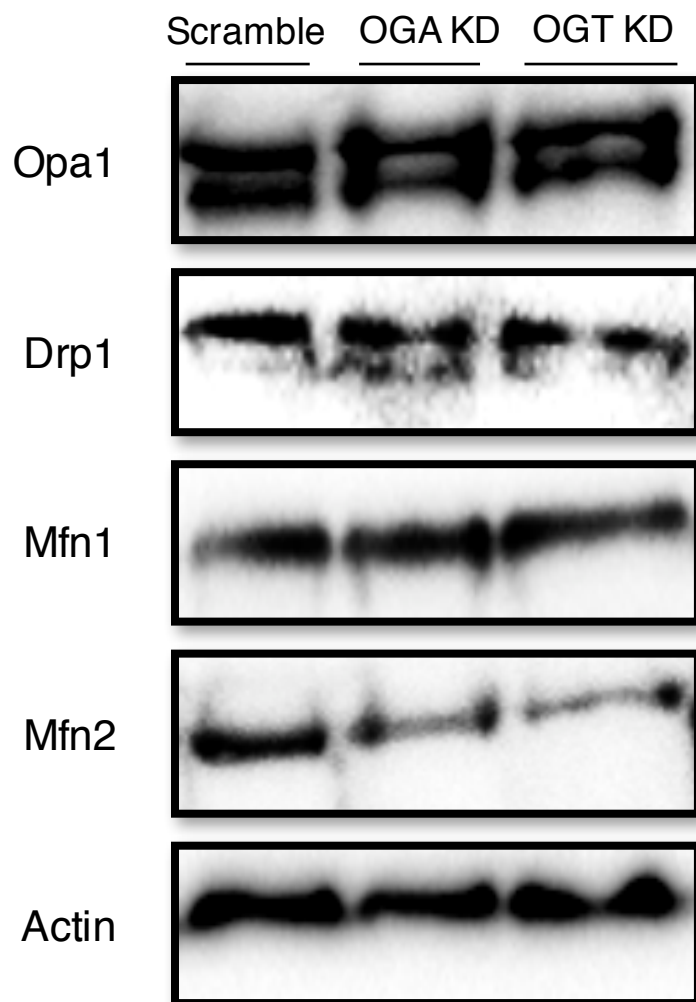


Figure 5.14: Stable knockdown of O-GlcNAcase and O-GlcNAc transferase has no effect on mitodynamin expression in differentiated PC12 cells. Scramble, OGA KD and OGT KD PC12 cells were plated and differentiated for 48 hours before being lysed with RIPA buffer and analysed for fusion mediating mitodynamins by western blot. No significant changes were observed in protein levels of Opa1, Drp1, Mfn1 or Mfn2 were observed. Images representative of n=3.

5.3.7 Effect of O-GlcNAcase and O-GlcNAc Transferase Knockdown on $\Delta\psi_m$ and ROS Production in Differentiated PC12 cells

Measurement of $\Delta\psi_m$ in knockdown cells using TMRM fluorescence as a measure provided some interesting results. Increasing cellular O-GlcNAc levels through knockdown of OGA resulted in an increase in $\Delta\psi_m$ in differentiated PC12 cells, while decreasing O-GlcNAc levels resulted in a significant decrease in $\Delta\psi_m$. Previous studies have shown that increasing O-GlcNAcylation through pharmacological treatment results in an decrease in $\Delta\psi_m$ (Gawłowski et al., 2012), while decreasing O-GlcNAcylation has resulted in an increase in $\Delta\psi_m$ (Banerjee et al., 2015). These results suggest that sustained alterations in O-GlcNAc levels may have a different effect or the effects of alterations in cellular O-GlcNAc levels may be different in different cell types. Dissipation of $\Delta\psi_m$ using an ionophore had the same effect in knockdown cells as in scramble cells (Fig 5.16E) indicating alterations in O-GlcNAc do not confer any protective effect in maintaining $\Delta\psi_m$. Inhibition of other complexes in the electron transport chain also resulted in a more gradual but sustained decrease in $\Delta\psi_m$ and a slight increase in the case of inhibition of ATP synthase (Fig 5.16B-E). However, there is a significant difference in the rate of $\Delta\psi_m$ when complex IV is inhibited by the addition of KCN in OGA knockdown cells, suggesting that there is some protective effect on inhibition by increased O-GlcNAcylation of complex IV.

ROS production was also measured in OGA and OGT knockdown cells and it was found that knockdown of OGT significantly increases ROS production in differentiated PC12 cells as shown in Fig 5.17B. When the initial rates of ROS production were measured it was also found that these too were significantly higher in OGT knockdown cells. Inhibition of OXPHOS in the knockdown cells showed that decreased levels of O-GlcNAc in these cells resulted in an increase in total ROS production as well as the initial rates of ROS production when complex I was inhibited with rotenone and complex IV was inhibited with KCN. It is interesting to note that inhibition of complex IV in these knockdown cells has the most obvious effects as a number of subunits of complex IV (including subunits encoded by mtDNA) have been shown to be O-GlcNAcylated (Ma et al., 2015).

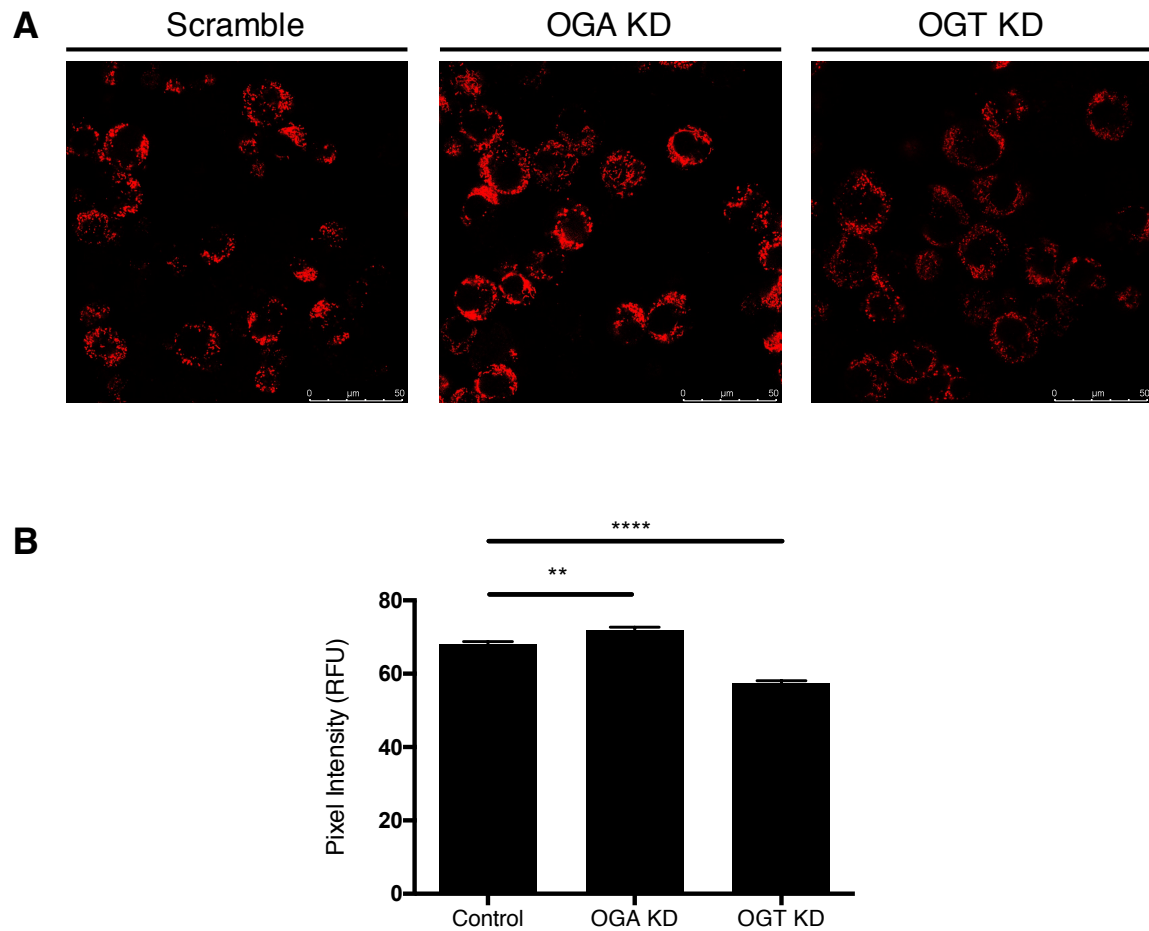


Figure 5.15: Knockdown of O-GlcNAcase increases mitochondrial membrane potential while stable knockdown of O-GlcNAc transferase decreases mitochondrial membrane potential in differentiated PC12 cells. (A) Images representative of TMRM fluorescence in scramble, O-GlcNAcase knockdown and O-GlcNAc transferase knockdown cells. (B) Analysis of pixel intensity using Imaris image analysis software shows significant difference in pixel intensity as a measure of $\Delta\psi_m$. OGA knockdown shows a significant increase in $\Delta\psi_m$ and OGT knockdown shows a significant decrease in $\Delta\psi_m$. Data presented as mean $\pm$ SEM; n=4. A one-way ANOVA followed by Dunnett's multiple comparisons test was performed and values which are significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

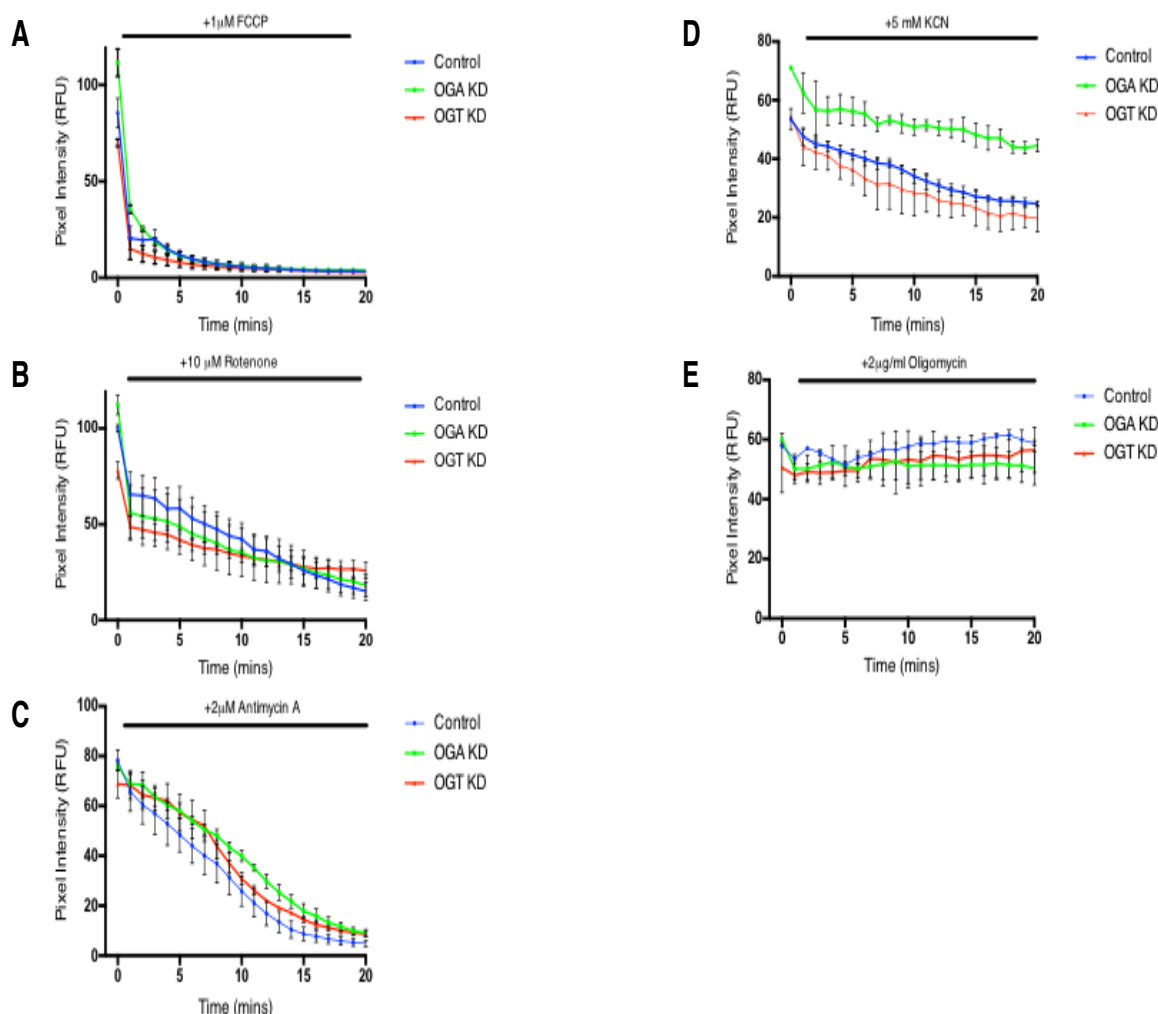


Figure 5.16: Inhibition of complexes I, III and IV in OGA and OGT knockdown cells significantly decreases mitochondrial membrane potential in O-GlcNAcase and O-GlcNAc transferase knockdown differentiated PC12 cells. Quantitative data shows the addition of an ionophore (A), a complex I inhibitor (B), a complex III inhibitor (C) or a complex IV inhibitor (D) significantly decreases $\Delta\psi_m$ in scramble, OGA knockdown and OGT knockdown differentiated PC12 cells over 20 minutes. Addition of oligomycin shows a slight increase in $\Delta\psi_m$ over the course of a 20 min experiment. Cells were incubated with 20 nM TMRM for 20 min before being washed with PBS and incubated in krebs buffer for the duration of imaging. Inhibitors were added after initial images were taken. Data presented as mean $\pm$ SEM, n=3.

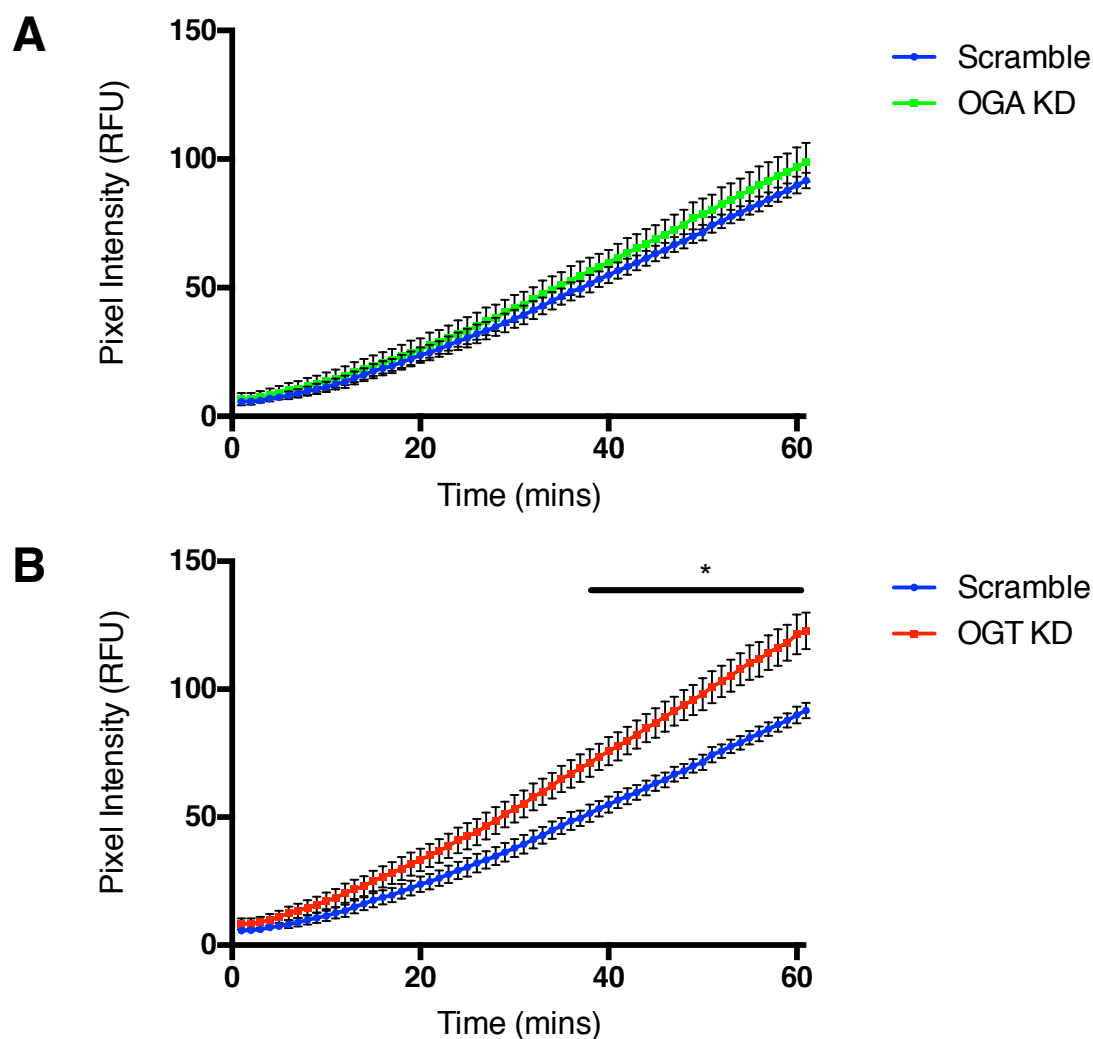


Figure 5.17: Knockdown of O-GlcNAc transferase increases ROS production in differentiated PC12 cells. Differentiated PC12 scramble, OGA knockdown (A) and OGT knockdown (B) were seeded in a black 96 well plate and differentiated for 48 hours before ROS production was measured using H₂DCFDA. Cells were washed with PBS and maintained in krebs buffer containing 5 μ M H₂DCFDA while fluorescence (excitation λ =485 and emission λ =530) was measured for 1 hour. ROS production was significantly increased in OGT knockdown cells. Data is expressed as relative fluorescence units $\pm$ SEM; n=3. An individual t-test was performed at each time point and values that are significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

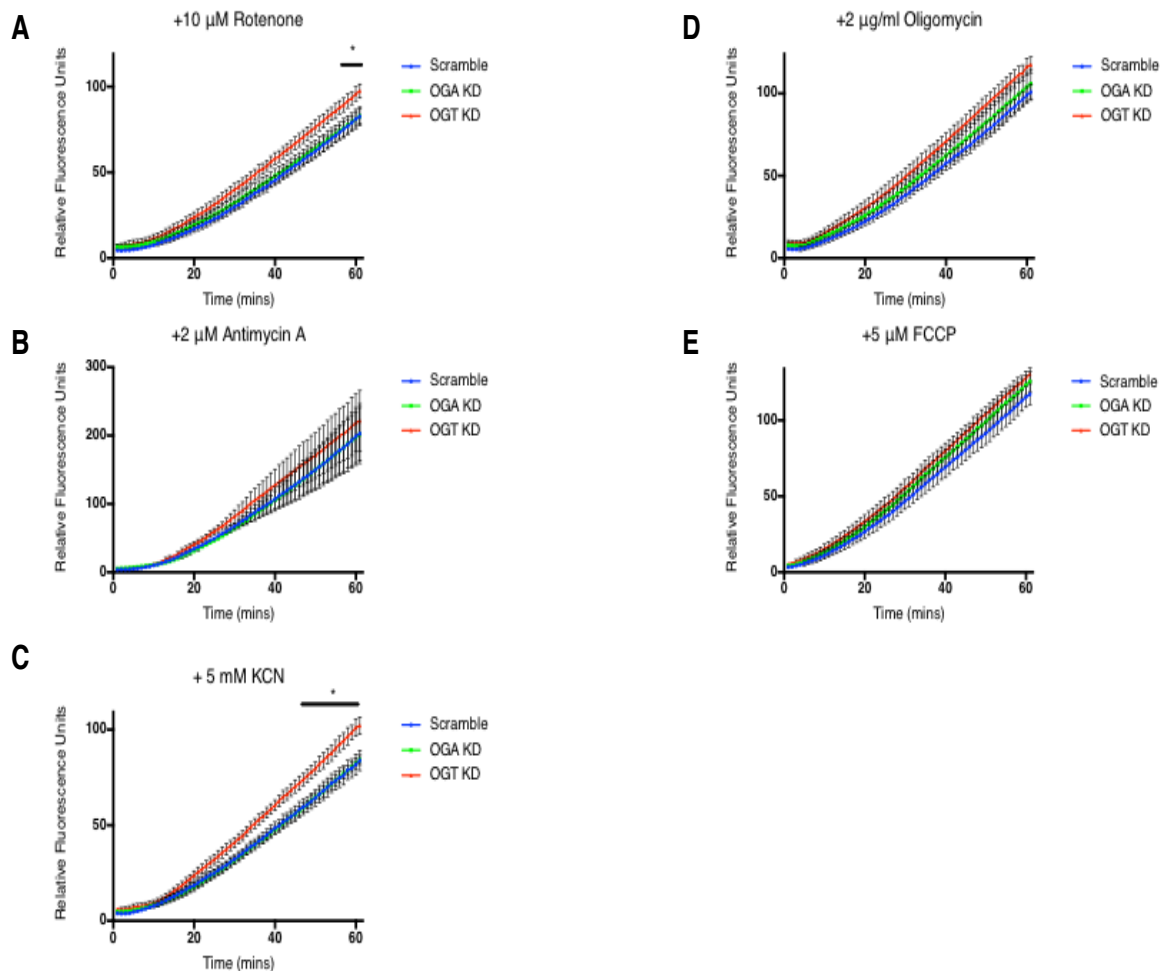


Figure 5.18: Inhibition of complex I and complex IV significantly increases ROS production in O-GlcNAc transferase knockdown cells compared to scramble cells. Differentiated PC12 scramble, OGA knockdown (A) and OGT knockdown (B) were seeded in a black 96 well plate and differentiated for 48 hours before ROS production was measured using H_2DCFDA . Cells were washed with PBS and maintained in krebs buffer containing 5 μ M H_2DCFDA and incubated with (A) 10 μ M rotenone, (B) 2 μ M antimycin A, (C) 5 mM potassium cyanide, (D) 2 μ g/ml oligomycin or (E) 5 μ M FCCP and fluorescence (excitation $\lambda=485$ and emission $\lambda=530$) was measured for 1 hour. ROS production was significantly increased in OGT knockdown cells treated with rotenone and KCN as compared to scramble cells treated with these inhibitors. Data is expressed as relative fluorescence units $\pm$ SEM; n=3. An individual t-test was performed at each time point and values that are significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

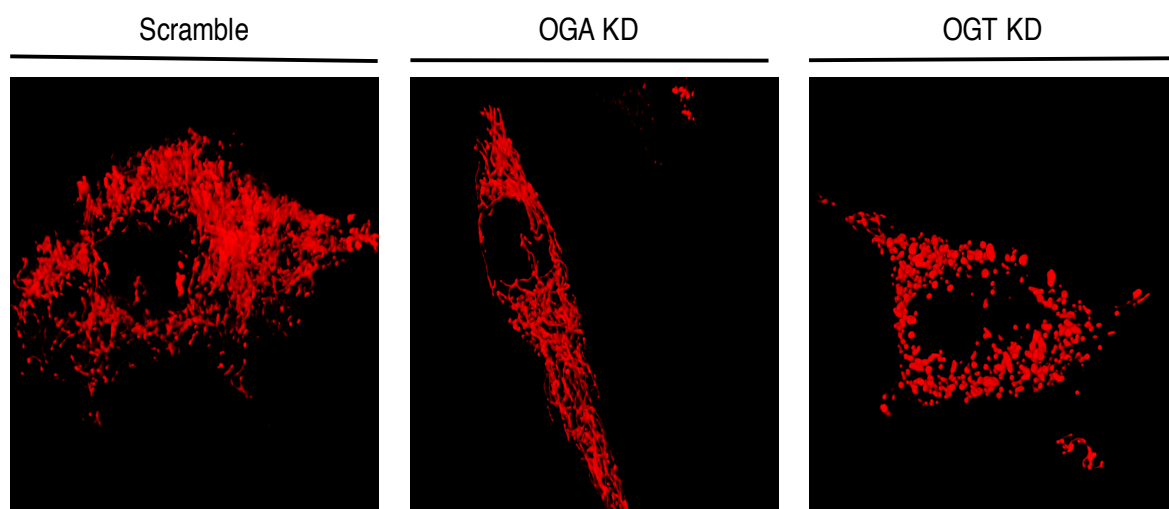


Figure 5.19. Mitochondrial morphology is significantly altered in O-GlcNAcase and O-GlcNAc transferase knockdown cells. Cells were transfected with DSRed2-mito plasmid and differentiated for 48 hours on poly-D-lysine coated glass coverslips before being fixed and mounted on slides. Scramble cells exhibited a varied mitochondrial network with a mixture of tubular and intermediate mitochondria. OGA knockdown cells displayed a mitochondrial network with elongated, tubular mitochondria while OGT knockdown cells had a fragmented mitochondrial network. Representative images; n=3.

5.3.8 Effect of O-GlcNAcase and O-GlcNAc Transferase Knockdown on Respiration in Differentiated PC12 cells

Respiration was measured using a seahorse XF24 extracellular flux analyser which determines oxygen consumption rates as well as extracellular acidification rates to estimate the amount of energy derived from glycolysis. Scramble, OGA and OGT knockdown cells were seeded in seahorse 24 well cell culture plates coated with poly-D-lysine and differentiated for 48 hours before media was replaced with seahorse media (also containing NGF). Seahorse cartridges were loaded with oligomycin, FCCP, rotenone/antimycin A and 2-deoxyglucose before plates were calibrated and the experiment began. As can be seen in Fig 5.20, basal oxygen consumption rates were increased in OGA knockdown cells and reduced in OGT knockdown cells. After the addition of oligomycin there was no difference in ATP derived respiration between any of the cells, but OGA knockdown cells again displayed a large significant increase in the maximal rate of respiration compared to scramble control (Fig 5.21B). In contrast, OGT knockdown cells displayed a significantly decreased maximal respiration rates indicating a greatly decreased spare capacity for oxygen consumption as a result of decreased cellular O-GlcNAcylation.

No significant differences were observed in ECARs indicating there is little difference in the glycolytic capacity of the cells (Fig 5.22). Fig 5.23 shows that OGA knockdown cells displayed a slightly reduced rate of glycolysis which is unsurprising considering the increased rate of oxygen consumption observed in these cells but interestingly there is no increase in glycolysis in OGT knockdown cells. These results indicate an important role for O-GlcNAcylation in cellular respiration.

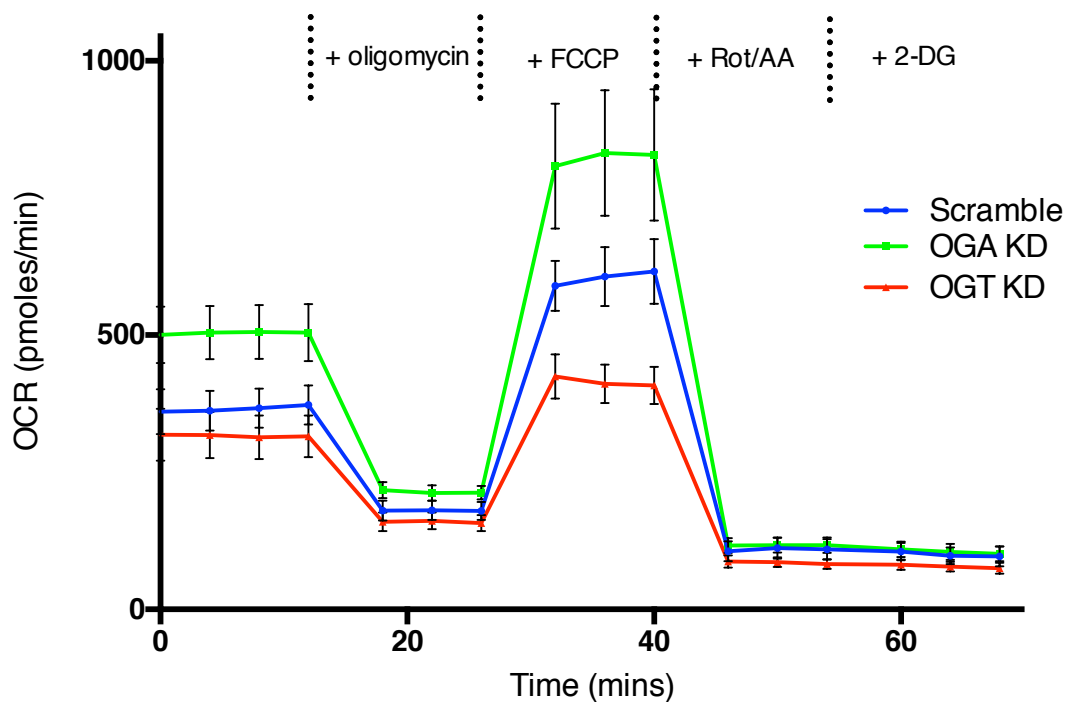


Figure 5.20: Basal and maximal respiration is increased in O-GlcNAcase knockdown differentiated PC12 cells and maximal respiration is decreased in O-GlcNAc transferase knockdown cells. Cells were differentiated in an XF24 seahorse cell culture plate coated with poly-D-lysine for 48 hours in RPMI 1640 before being switched to seahorse medium 45 min prior to the start of the experiment. Seahorse cartridges were loaded with inhibitors (oligomycin, FCCP, rotenone + antimycin A and 2-deoxyglucose). OGA knockdown cells displayed increased basal and maximal oxygen consumption rates, indicative of an increased rate of respiration. OGT knockdown cells displayed a slightly decreased basal oxygen consumption rate and a significantly reduced maximal oxygen consumption rate. Data presented as mean $\pm$ SEM; $n=4$.

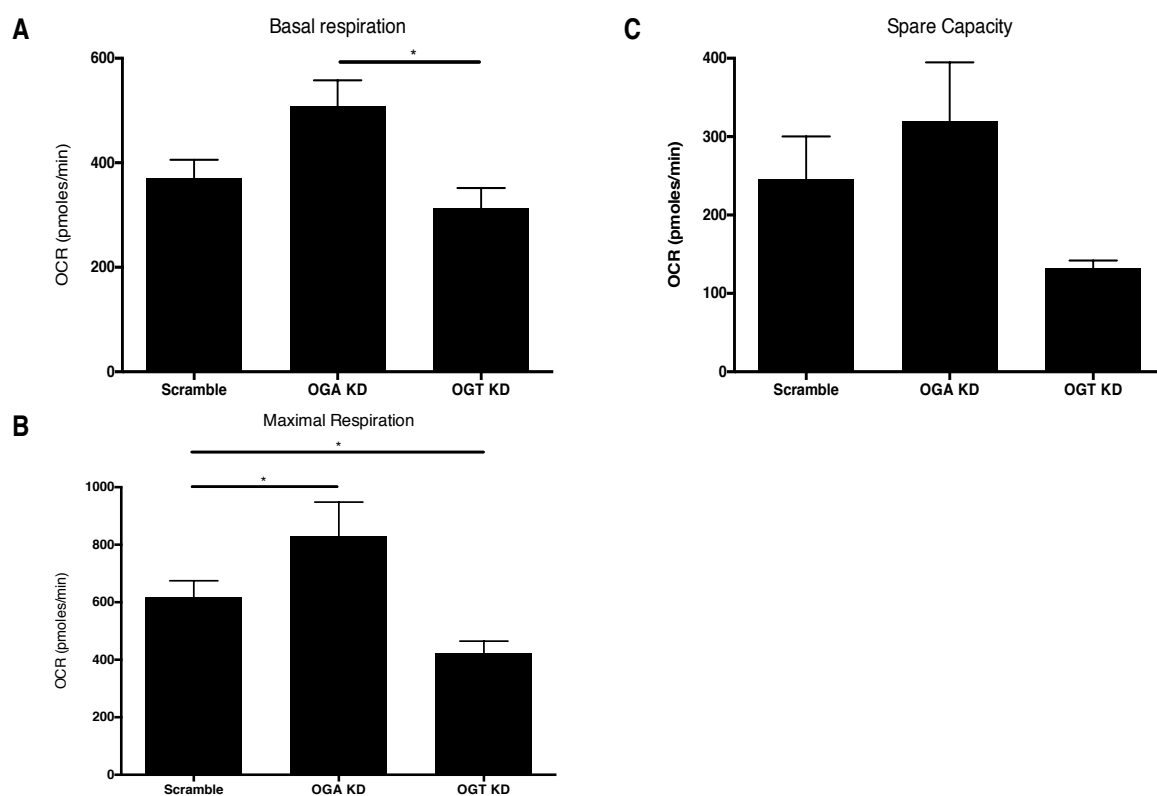


Figure 5.21: Knockdown of O-GlcNAcase significantly increases and knockdown of O-GlcNAc transferase significantly decreases maximal oxygen consumption rates in differentiated PC12 cells. Oxygen consumption rates were measured using a seahorse XF24 extracellular flux analyzer. Data presented as mean $\pm$ SEM; n=4. A one-way ANOVA followed by Dunnett's multiple comparisons test was performed and values which are significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

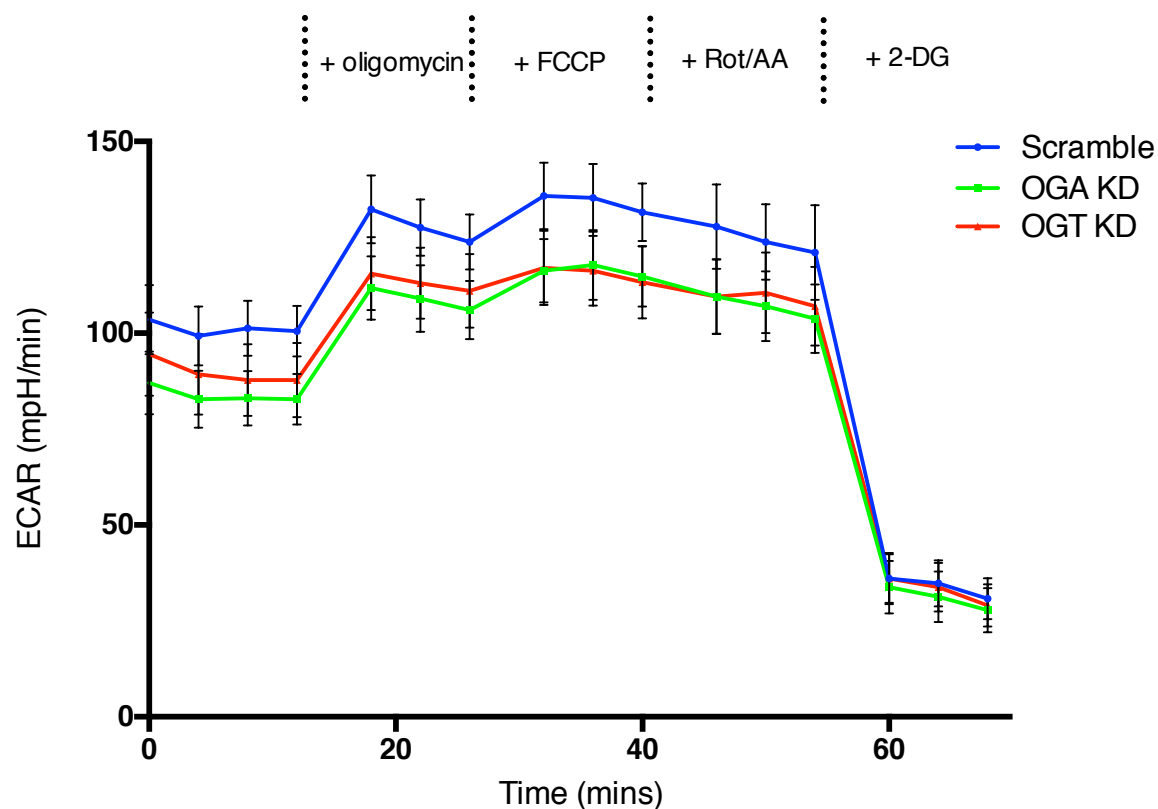


Figure 5.22: O-GlcNAcase or O-GlcNAc transferase knockdown does not significantly affect extracellular acidification rates in differentiated PC12 cells. Cells were differentiated in an XF24 seahorse cell culture plate coated with poly-D-lysine for 48 hours in RPMI 1640 before being switched to seahorse medium 45 min prior to the start of the experiment. Seahorse cartridges were loaded with inhibitors (oligomycin, FCCP, rotenone + antimycin A and 2-deoxyglucose). No significant differences in extracellular acidification rates, as a measure of glycolysis, were observed as compared to scramble control. Data presented as mean $\pm$ SEM; n=4.

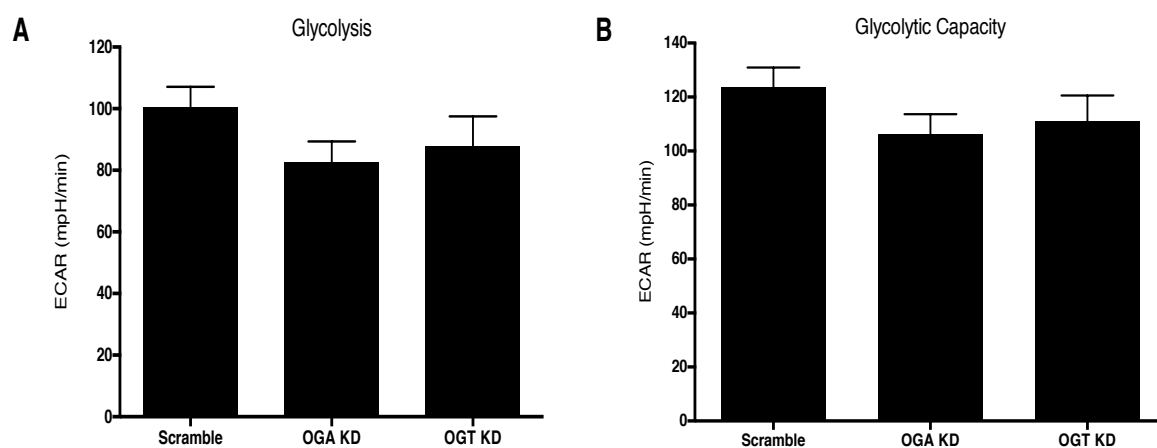


Figure 5.23: O-GlcNAcase or O-GlcNAc transferase knockdown does not significantly affect extracellular acidification rates in differentiated PC12 cells. Extracellular acidification rates were measured using a seahorse XF24 extracellular flux analyser. Data presented as mean $\pm$ SEM, n=4. A one-way ANOVA followed by Dunnett's multiple comparisons test was performed and values which are significantly different from control are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

5.3.9 Effect of O-GlcNAcase and O-GlcNAc Transferase Knockdown on Electron Transport Chain Complex Activity in Differentiated PC12 cells

Complex I, complex II/III and complex IV activities were measured in scramble, OGA and OGT knockdown differentiated PC12 cells which had been homogenised and freeze-thawed 3 times. Complex I activity was significantly increased in both OGA knockdown cells as compared to scramble, with complex I activity showing a 50% increase compared to the rate of scramble control in OGA knockdown cells (Fig 5.24). There was no significant difference in complex II/III activity (Fig. 5.25) and complex IV activity was decreased in OGA knockdown cells but no significant difference was seen in OGT knockdown cells (Fig 5.26). Taken together, these results indicate that complex I activity is strongly affected by increased levels of O-GlcNAcylation while complex IV shows lower rates of activity when there is increased cellular O-GlcNAc.

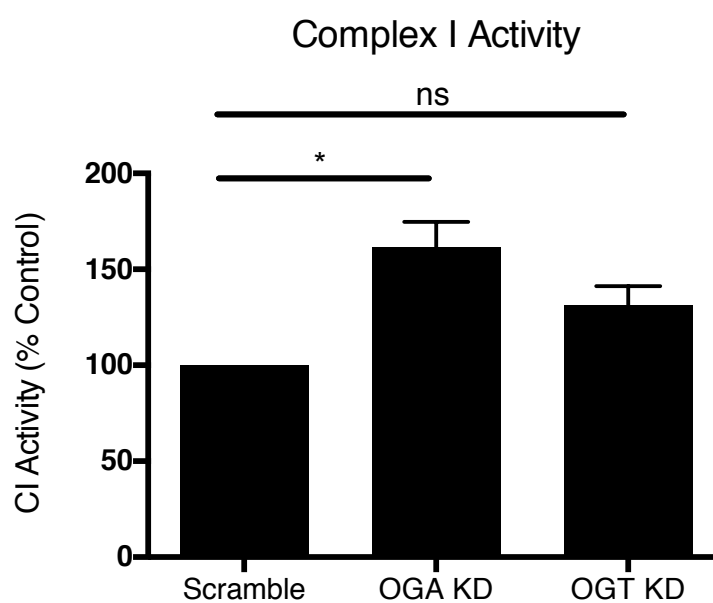


Figure 5.24: Complex I activity is significantly increased in homogenates from O-GlcNAcase knockdown differentiated PC12 cells. Scramble, OGA and OGT knockdown cells were differentiated for 48 hours before being washed, homogenized and freeze-fractured three times. Complex I activity was measured with a spectrophotometric assay following the oxidation of NADH at $\lambda=340$ nm at 37 °C. Data is expressed as percent of scramble $\pm$ SEM; n=3. A Student's t-test was performed with scramble for each knockdown and values which were significantly different from scramble are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

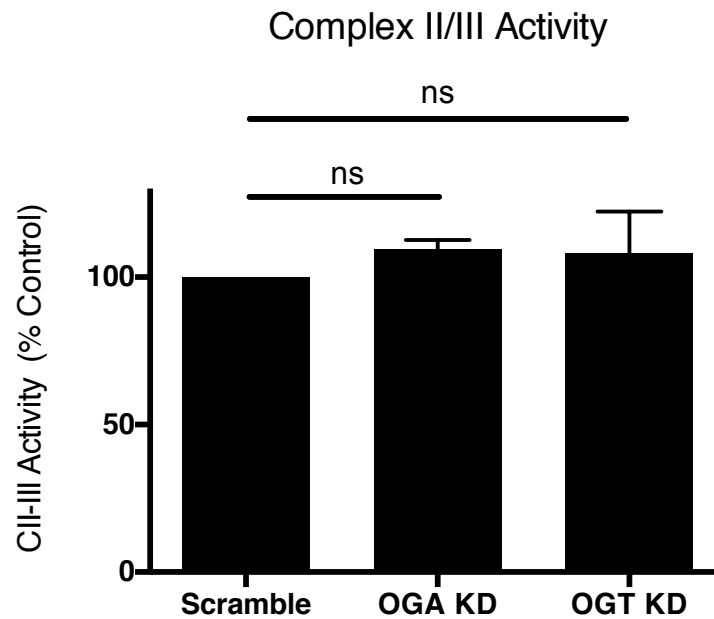


Figure 5.25: Complex II/III activity is not significantly changed in homogenates from O-GlcNAc transferase or O-GlcNAcase knockdown PC12 cells. Scramble, OGA and OGT knockdown cells were differentiated for 48 hours before being washed, homogenized and freeze-fractured three times. Complex II/III activity was measured with a spectrophotometric assay following the reduction of cytochrome *c* at $\lambda=550$ nm at 37 °C. Data is expressed as percent of scramble $\pm$ SEM; n=3. A Student's t-test was performed with scramble for each knockdown and values which were significantly different from scramble are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

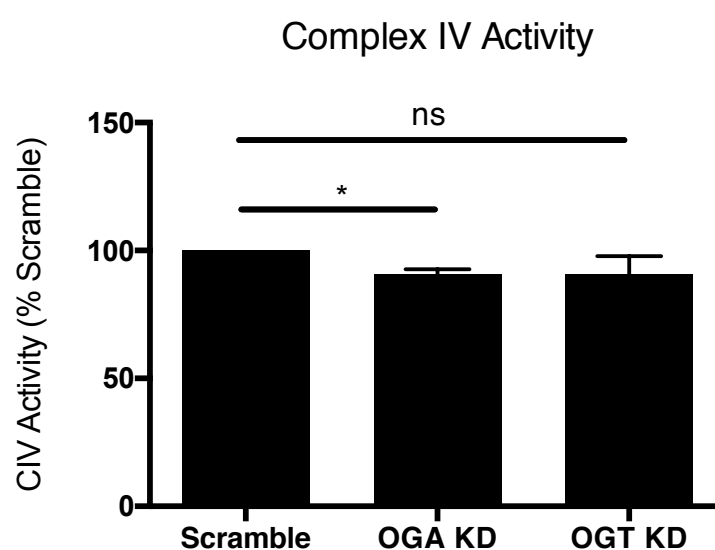


Figure 5.26: Complex IV activity is significantly decreased in homogenates from O-GlcNAcase knockdown PC12 cells. Scramble, OGA and OGT knockdown cells were differentiated for 48 hours before being washed, homogenized and freeze-fractured three times. Complex IV activity was measured with a spectrophotometric assay following the oxidation of cytochrome *c* at $\lambda=550$ nm at 37 °C. Data is expressed as percent of scramble $\pm$ SEM; n=3. A Student's t-test was performed with scramble for each knockdown and values which were significantly different from scramble are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

	+ Thiamet G	+ TT04	OGA KD	OGT KD
Viability	Unchanged	Decreased	Unchanged	Unchanged
Mitochondrial Fusion Rates	Decreased	Unchanged	Unchanged	Unchanged
$\Delta\Psi_m$	Unchanged	Unchanged	Increased	Decreased
ROS	Unchanged	Unchanged	Unchanged	Increased
Mitochondrial Morphology	-	-	Elongated	Fragmented
Respiration	-	-	Increased	Decreased
Glycolysis	-	-	Unchanged	Unchanged
Complex I	-	-	Increased	Unchanged
Complex II/III	-	-	Unchanged	Unchanged
Complex IV	-	-	Decreased	Unchanged

Figure 5.27 – Summary of Chapter 5 results. Table showing the effect of increasing or decreasing O-GlcNAcylation by pharmacological inhibition or siRNA mediated knockdown of the enzymes involved in O-GlcNAc cycling. Thiamet G and knockdown of OGA increase global cellular O-GlcNAc levels, while TT04 and knockdown of OGT decrease global cellular O-GlcNAc levels.

5.4 Discussion

O-GlcNAcylation is a post-translational modification that is ubiquitous throughout the body in eukaryotes and regulates many cell functions. Recent evidence has established that expression levels of OGA and OGT are especially high in the brain, suggesting an important role for O-GlcNAcylation in neuronal function and potentially a high rate of O-GlcNAc turnover. This area of the field has expanded rapidly in recent years as a number of O-GlcNAc modifications have been implicated in neurodegenerative disease, in particular AD and PD, and specifically protein aggregation in these diseases. Some inhibitors of O-GlcNAcylation have shown therapeutic benefit in mouse models of AD by reducing tau protein aggregation (Liu et al., 2004). Much of the work carried out thus far has focused on the effects of the O-GlcNAc modification on cellular and mitochondrial function in cardiac myocytes because of the links between diabetes and ischemia. Results have shown that altered levels of O-GlcNAc have effects on mitochondrial function in these cells with changes in ETC activities, ATP production and oxygen consumption observed. However, the effects of O-GlcNAcylation on mitochondrial dynamics and mitochondrial function in the brain have remained largely unexplored. Here, we use a neuronal cell model to investigate the effects of short term inhibition of OGA and OGT on mitochondrial dynamics. We also investigate the effects of knockdown of the enzymes responsible for O-GlcNAc cycling on mitochondrial dynamics and the functions of individual components of the ETC, as well as cellular bioenergetics.

OGA was pharmacologically inhibited by thiamet G and the gene was knocked down by shRNA, both cases resulting in an increase in cellular O-GlcNAc levels in differentiated PC12 cells (Figs 5.1 and 5.11). In a similar manner OGT was inhibited using TT04 and knocked down using shRNA, and both techniques resulted in a decrease in overall cellular O-GlcNAc. These experiments showed that the enzymes are active in differentiated PC12 cells and could be inhibited to alter levels of O-GlcNAcylation. It was also shown that knockdown of the enzymes had no effect on cell viability but pharmacological inhibition of OGT did slightly

reduce cell viability after a 4 hour treatment indicating there may be some toxicity associated with the drug. These results confirm that OGA inhibition by thiamet G can increase O-GlcNAc levels in differentiated PC12 cells as first reported by Yu et al. (2012). It has not been shown to date that cells treated with TT04 can reduce O-GlcNAc levels in differentiated PC12 cells but we confirm that this is the case as has been reported in other cell types and that 2 μ M treatment decreases cell viability by ~15%.

There is some evidence to support a role for O-GlcNAcylation in mitochondrial dynamics and morphology but there has been little published on the effect of varying levels of O-GlcNAc on mitochondrial fusion *per se*. Pekkurnaz et al. (2014) found that increased levels of O-GlcNAcylation in rat hippocampal neurons decreased mitochondrial motility which would be expected to decrease mitochondrial fusion by decreasing the number of mitochondrial contact events. In addition to this, increased O-GlcNAcylation of Drp1 at T585 decreases phosphorylation at S637, which increases the fission activity of the protein and augments the level of the GTP-bound active form (Gawlowski et al., 2012). To assess the effect of increased O-GlcNAcylation on mitochondrial fusion in differentiated PC12 cells, cells were pre-treated with 100 nM thiamet G for 16 hours before carrying out the confocal fusion assay. As seen in Fig 5.4, this resulted in a decreased rate of mitochondrial fusion, likely as a result of decreased mitochondrial motility. Similarly, treatment with PUGNAc or alloxan had the same effect on mitochondrial fusion but these compounds were not used in further experiments due to concerns about their toxicity as the concentrations used were high. Interestingly, increasing O-GlcNAc levels by knocking down OGA had no effect on mitochondrial fusion as seen in Fig 5.12, despite the fact that mitochondrial morphology appeared to be elongated and tubular. As these are stable knockdowns, it may be the case that mitochondrial fusion was increased at an earlier time point and normal fusion has returned at this point in order to maintain the elongated mitochondrial network. Decreasing O-GlcNAc levels by pharmacological inhibition of OGT by TT04 had no effect on mitochondrial fusion, shown in Fig 5.6, which was a surprising result as it was thought this may increase

mitochondrial motility and subsequently increase the number of fusion events. However, a slight increase was seen when O-GlcNAc levels were decreased by knockdown of OGT (Fig 5.13), suggesting motility may have been increased. It would be interesting to measure motility speeds in the anterograde or retrograde direction in neurites to determine if the knockdowns have altered motility or some other part of the fusion machinery.

It should be noted that the effects produced by decreased activity of the enzymes may not be related to any change in mitochondrial motility. There are regularly new discoveries of O-GlcNAc sites on proteins and a wide variety of mitochondrial proteins have already been shown to be O-GlcNAcylated so it would be naïve to assume that the global changes in O-GlcNAcylation seen are as a result of the activity of one protein. If we were to take Drp1 for example, one would expect more fissioned, punctate mitochondria after treatment with thiamet G, as Drp1 activity should be increased. However, instead we see an elongated mitochondrial phenotype which is especially obvious in the OGA knockdown, but can also be seen after short term thiamet G treatment. In fact, as seen in Fig 5.19, knockdown of OGT which decreases O-GlcNAc levels gives a fragmented mitochondrial network with a more punctate phenotype. Other groups have reported that both increased and decreased O-GlcNAcylation through knockdown or overexpression of OGT have led to a fragmented mitochondrial network (Sacoman et al., 2017, Makino et al., 2011). This implies that the effect of O-GlcNAcylation on mitochondrial morphology may be cell-type specific or effects may be exacerbated in cell types which have high expression of mitodynamin and/or high fusion rates. The different effects seen suggest that O-GlcNAc cycling is a dynamic process that changes in response to the cellular microenvironment and therefore may be a regulator of many different pathways. Therefore, targeting O-GlcNAc levels may be time dependent and have a ripple effect throughout the cell.

Other studies have shown that decreasing O-GlcNAcylation pharmacologically by inhibiting OGT strengthens $\Delta\psi_m$ while treatment with thiamet G and PUGNAc

significantly decreases $\Delta\psi_m$ (Gawlowski et al., 2012, Banerjee et al., 2015). Here we see no significant differences in $\Delta\psi_m$ with thiamet G or TT04 treatment (Fig 5.7), although the trend observed is opposite to those observed in the literature. The knockdown experiments agree with the results of pharmacological inhibition of OGA and OGT in differentiated PC12 cells as OGA knockdown results in a significant increase in $\Delta\psi_m$ in differentiated PC12 cells and OGT knockdown significantly reduces $\Delta\psi_m$ (Fig 5.15). As viability is unaffected in the knockdown cells, it is not possible that the decrease in $\Delta\psi_m$ observed is due to apoptosis, which is often associated with $\Delta\psi_m$ decreases. The reduction in $\Delta\psi_m$ is understandable considering the increases in ROS (Fig 5.17) and decrease in respiration (Fig 5.20) observed in the OGT knockdown cells which will be further discussed below. However, it conflicts with the observations that there is no change in the activity of the ETC complexes (Fig. 5.24, 5.25, 5.26). Opa1 has been shown to be O-GlcNAcylated (Makino et al., 2011) and has also been shown to be important in maintaining cristae tightness and subsequently super-complex structure (Frezza et al., 2006, Cogliati et al., 2013). It is possible that decreased O-GlcNAcylation of Opa1 alters Opa1 activity, reducing its ability to maintain cristae tightness and to maintain supercomplexes in their optimum structure. This would have the effect of reducing the overall respiratory capacity of the cell and reduce mitochondrial membrane potential despite the reduction in O-GlcNAcylation having no effect on the activity of each complex when assayed individually. This hypothesis gains further support when one observes the morphology of the mitochondria in the OGT knockdown cells which appear punctate and fragmented. It would be useful to carry out electron microscopy of the knockdown cells to observe any variations in cristae structure and blue native gels to examine any difference in supercomplexes between the OGA and OGT knockdown PC12 cells.

Alternatively, the higher levels of ROS produced in the OGT knockdown cells, as shown in Fig 5.17, may explain the conundrum between decreased respiration rates and unchanged ETC complex activities. Higher levels of ROS produced overall and when complex I and IV are inhibited suggest that these mitochondria

may be quite “leaky” resulting in a decreased $\Delta\psi_m$. This would also explain the lower levels of respiration seen in Fig 5.20, as mitochondria from OGA knockdown cells are likely to be in a physiological state somewhere closer to state 3 respiration, while those from OGT knockdown cells may be respiring in state 4. It would be interesting to isolate mitochondria from the cells and measure oxygen consumption rates in a Seahorse machine or using a Clark electrode to determine the respiratory control ratios.

H₂DCFDA was used to measure changes in the rate of ROS production in cells with altered O-GlcNAc levels and as can be seen in Fig 5.8, pharmacological inhibition of OGA and OGT had no effect on ROS production in differentiated PC12 cells. However, knockdown of OGT did produce an increase in overall ROS production and the initial rate of ROS production (Fig 5.17). This effect has been reported before by Ngoh et al. (2011) who found that a decrease in O-GlcNAc levels was correlated with an increase in ROS production under hypoxic conditions. Here, we see increased rates of ROS production compared to scramble cells under basal conditions and also when complex I and complex IV are inhibited. Despite a number of associations between increased ROS and increased O-GlcNAcylation (Du et al., 2000), our data shows that a decreased level of O-GlcNAcylation increases ROS, likely because of proton leakage at complex I and complex IV. This data suggests that O-GlcNAcylation is important for maintaining mitochondrial coupling and maintaining ROS production at normal levels in a neuronal model.

There is also somewhat conflicting evidence on the effect of altering O-GlcNAc levels on respiration rates. Tan et al. (2014) showed that overexpression of both OGA and OGT resulted in an overall decrease in respiration rates while others have reported that thiamet G treatment (Ma et al., 2015) and OGT knockdown (Sacoman et al., 2017) increase oxygen consumption rates. As seen in Fig. 5.20, our data shows an increase in basal and maximal respiration rates in OGA knockdown cells. Conversely, OGT knockdown results in a decreased rate of basal and maximal respiration in comparison to scramble cells. Again, it must be

noted that much of the previous work in this area has been carried out in cardiac cells and so seems to highlight the cell specific nature of O-GlcNAc cycling. It is unclear if the altered rates of respiration are due to changes in the activities of the ETC complexes or perhaps more likely due to alterations in the O-GlcNAcylation of a metabolic master regulator.

Due to the large number of mitochondrial and cellular proteins which are O-GlcNAcylated it is obvious there is no single role for increased or decreased O-GlcNAcylation in the metabolism of this neuronal model. Instead, it appears likely that alterations in O-GlcNAc cycling can have effects on individual complexes (such as those of the ETC or the fission/fusion machinery) and also have effects on overall neuronal cell respiration. An interesting hypothesis by Banerjee et al. (2015) suggested that OGT inhibition may be more specific to mitochondrial metabolism and OGA inhibition affects the overall cellular metabolic rate. The results presented in our study with knockdown of the enzymes strengthens this view. OGT knockdown significantly alters the activity of all the ETC complexes while maintaining a significantly lower oxygen consumption rate while OGA knockdown has more subtle effects on the ETC complexes while significantly increasing oxygen consumption rates. It would be interesting to measure overall ATP production in the two knockdown cell lines as one would expect significant changes based on the results obtained.

Overall, it may be concluded that the cycling of O-GlcNAc on and off target proteins may be the most important aspect in maintaining cellular homeostasis. The large shifts seen here after long term knockdown of the enzymes are somewhat replicated in pharmacological inhibition in PC12 cells and in synaptosomes and non-synaptic mitochondria (unpublished data from the Davey lab) indicating short term increases and decreases in O-GlcNAcylation levels have an important physiological role. Elucidating the effects of these changes on specific proteins will provide us with a more detailed picture of their role in the pathogenesis of diabetes and neurodegenerative disorders.

Chapter 6

Metabolic Labelling of the Cell
Surface Glycocalyx of Primary
Cortical Neurons

6.1 Introduction

Glycosylation is a common of all post-translational modification, involving the addition of a carbohydrate chain or glycan to a protein or lipid resulting in a great diversity of carbohydrate conjugates bonds. Protein glycosylation has major impacts on their structure and function, and interactions of glycoproteins with carbohydrate binding proteins (lectins) modulate many important biological processes. Here, we investigate the glycocalyx and composition of the cell-surface glycome of primary cortical neurons and identify the primary types of glycosylation on the surface of these neuronal cells, which include a variety of proteins including neurotransmitter receptors, ion channels and adhesion proteins.

6.1.1 Glycosylation and Neurodegeneration

Mutations in glycosyltransferases and other components of the glycosylation system often result in a neurological phenotype, with substantial evidence pointing to a role for altered glycosylation in neurodegeneration. It is interesting to note that the majority of glycoproteins found in the brain and cerebrospinal fluid (CSF) are distributed to extracellular and membrane compartments, indicating that the cell surface glycocalyx is an important and largely unexplored area of research (Hwang et al., 2010). Acetylcholinesterase (AChE), for example is a glycoprotein, which terminates neurotransmission at cholinergic synapses by hydrolyzing acetylcholine and is often targeted by AChE inhibitors in Alzheimer's Disease (AD) in order to increase synaptic transmission in cholinergic neurons, of which there are many in the hippocampus and other brain regions associated with AD. AChE has been shown to have an altered glycosylation pattern in post-mortem brain tissue and cerebrospinal fluid (CSF) of AD patients, yet this phenomenon has not been seen in any other neurodegenerative disease (Sáez-Valero et al., 2000). Further studies have found that a related esterase, butyrylcholinesterase is also glycosylated in a unique manner in the CSF of patients with AD (Saez-Valero and Small, 2001).

In Parkinson's Disease (PD), a classification of glycoproteins by gene ontology analysis has shown that several overlapping glycoproteins between human CSF and brain tissue are linked to PD pathogenesis, including neuronal pentraxin II, which is involved in neuronal plasticity, regulation of AMPA receptors and is upregulated 8-fold in PD (Moran et al., 2008). The sodium channel $\beta 4$ subunit has four glycosylation sites in the extracellular region subunit and its expression has also been shown to be increased with PD progression. By overexpressing $\beta 4$ -WT and $\beta 4$ -4 MUT (in which the subunit is deglycosylated) plasmids in Neuro2a cells, showed that the mutant accelerated neurite extension and increased the number of filopodia-like protrusions, suggesting a role for altered glycosylation of the $\beta 4$ subunit in the pathogenesis of PD (Zhou et al., 2012). Evidence of altered glycoproteins have also been found in HD, ALS and MS. In HD, altered expression of the genes encoding glycosyltransferases that are involved in the synthesis of gangliosides results in an imbalance in ganglioside metabolism (Desplats et al., 2007). Furthermore, in ALS patients there is evidence of Golgi disruption in motor neurons suggesting alterations in the glycosylation patterns of secretory proteins (Gonatas et al., 2006) and patients have been shown to have high levels of sialylated glycans and low levels of fucosylated glycans as compared to healthy controls (Edri-Brami et al., 2012). This evidence suggests that glycoproteomic alterations are a mechanistic part of disease progression in a variety of neurodegenerative diseases and it is therefore beneficial and therapeutically important to study and identify the specific glycoconjugates of the glycocalyx of neurons.

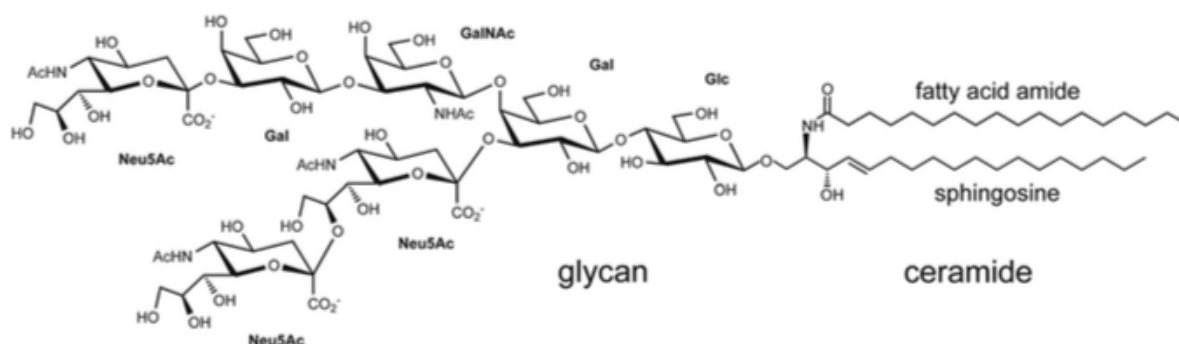


Figure 6.1 – Trisialoganglioside GT1b. Image showing the structure of trisialoganglioside which is one of the four major mammalian brain gangliosides. It's structure is more complex than the others and is linked to a ceramide via glucose. Taken from Schnaar (2016)

6.1.2 GlcNAc, GalNAc and ManNAc

GlcNAc, GalNAc and ManNAc are simple hexosamine sugars that are most commonly present in *N*- and *O*-linked glycosylation. All *N*-glycans share a common pentasaccharide core, GlcNAc₂Man₃, while *O*-glycans have a number of different core structures, beginning with the addition of GalNAc to a serine or threonine residue. Other carbohydrate structures including GlcNAc, GalNAc, mannose, fucose and galactose extend the glycan chain which is often capped with a sialic acid, giving the polysaccharide chain a negative charge and a large degree of complexity. ManNAc is different than GlcNAc and GalNAc as it is not a natural acetylated form of mannose but can be synthetically made and directly incorporated into the sialic acid, neuraminic acid (Neu5Ac) (Wiederschain, 2009).

Click-chemistry using synthetic azido forms of GlcNAc, GalNAc and ManNAc has been shown to be a powerful technique to study glycosylation in mammalian cells (Saxon and Bertozzi, 2000). Using a “bio-orthogonal chemical reporter” strategy where the monosaccharide substrate is modified with a functional azido group, followed by administration to cells and incorporation into cellular glycans, glycosylation and glycoprotein biology can be studied. GlcNAc, GalNAc and ManNAc are synthetically modified by the addition of an organic azide (R-N₃) forming the modified sugars GlcNAz, GalNAz and ManNAz. Once these sugars are incorporated into cellular glycans, the labelled glycans are reacted with a probe linked to a complementary bio-orthogonal functional alkyne group under certain conditions. The two functional groups have mutually selective chemical reactivity ensuring only the labelled glycans are targeted for detection/visualization (Laughlin et al., 2006, Prescher and Bertozzi, 2006). Following incorporation into cells ManNAz is converted metabolically to SiaNAz (Fig 6.2) whose natural counterpart is sialic acid, a frequent terminal monosaccharide found on a wide variety of mammalian glycoconjugates. GalNAz is taken up the cells and substitutes GalNAc, which is the core residue of many mucin-type *O*-linked glycans as well as chondroitin sulfate proteoglycans, while GlcNAz labels many intracellular proteins normally bearing *O*-GlcNAc residues as well as *N*- and *O*-linked glycans produced in the endoplasmic reticulum and Golgi (Fig 6.3).

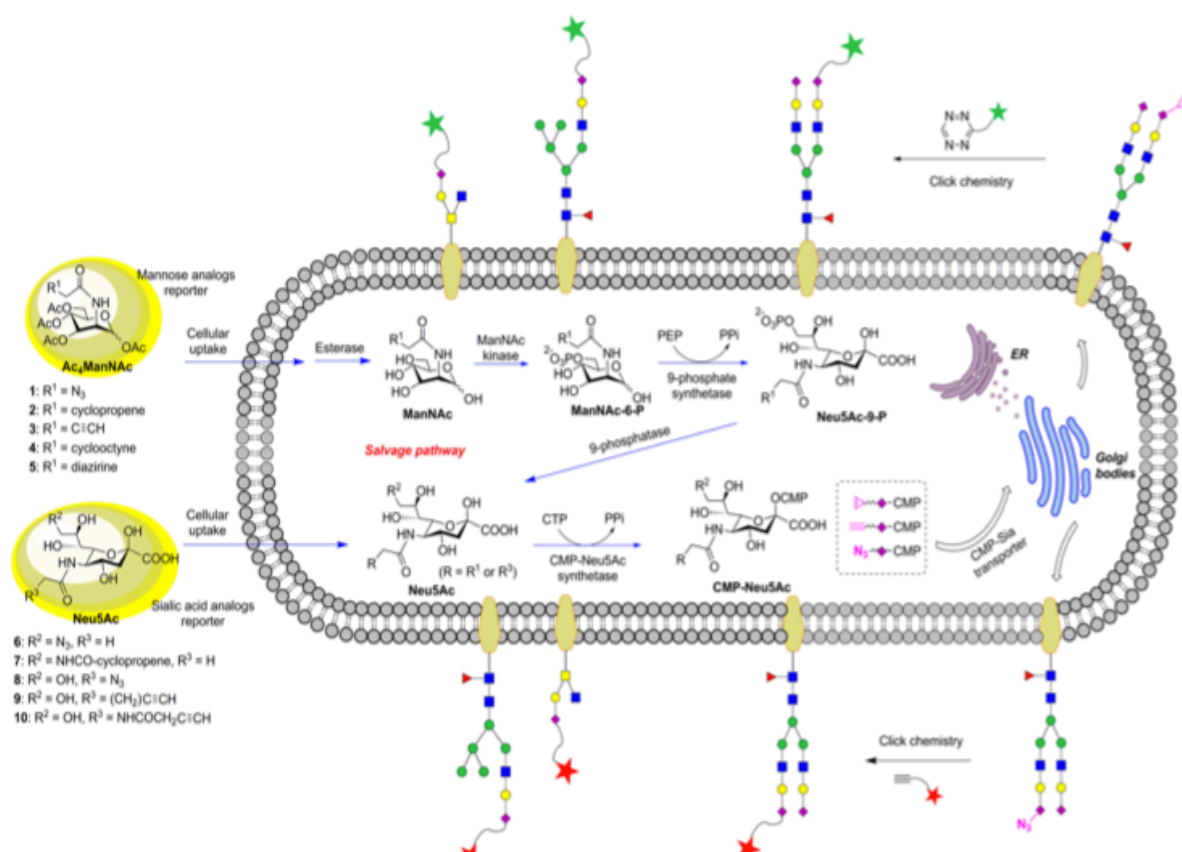


Figure 6.2 – Metabolic cell surface incorporation of ManNAz using the salvage sialic acid biosynthetic pathway. ManNAz carrying a chemical reporter (azide group) is metabolized to form the cytidine monophosphate sialic acid (CMP-Sia) derivative, which is then incorporated into the biosynthesis of glycoconjugates. The chemical reporter is then visualized by a biorthogonal click reaction in which an alkyne conjugated to an Alexa 488 fluorophore. Taken from Tan and Wang (2017).

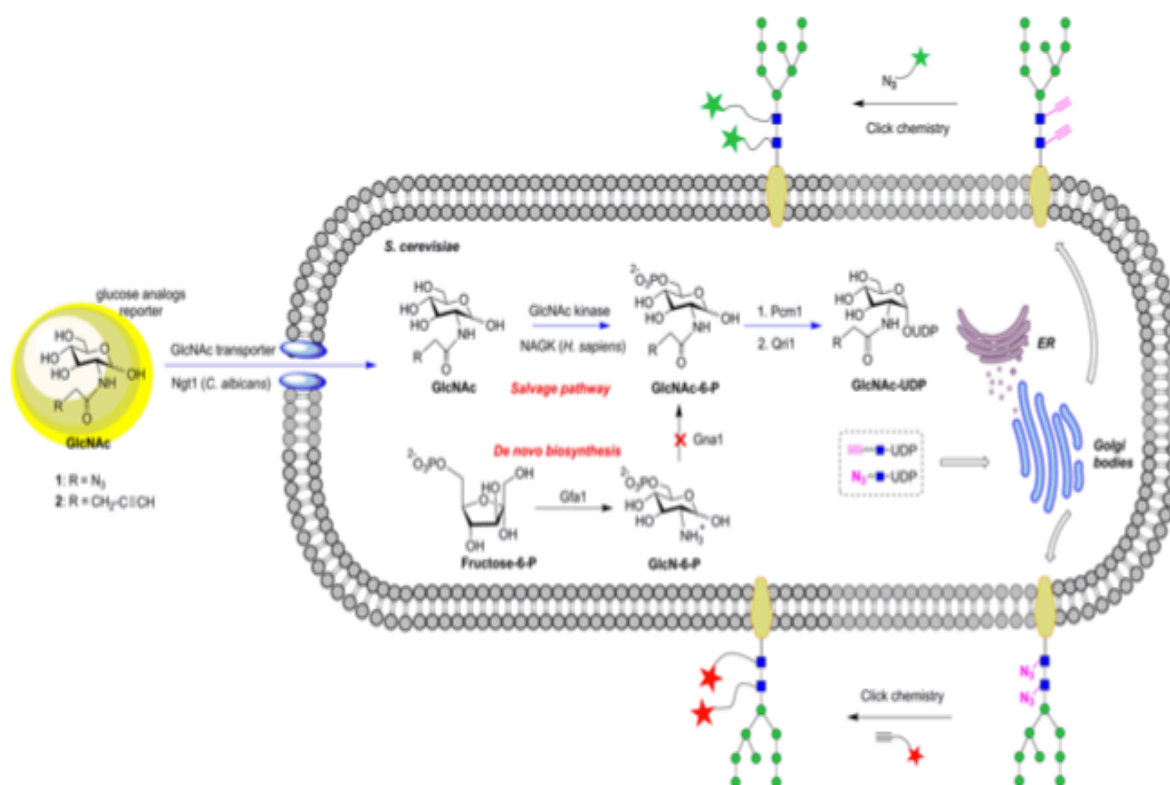


Figure 6.3 – Metabolic cell surface incorporation of GlcNAz using the salvage GlcNAc biosynthetic pathway. GlcNAz carrying a chemical reporter (azide group) is metabolized to form the uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAz), which is then incorporated into the biosynthesis of glycoconjugates. The chemical reporter is then visualized by a biorthogonal click reaction in which an alkyne conjugated to an Alexa 488 fluorophore. Taken from Tan and Wang (2017).

6.1.3 Inhibitors of Glycosylation

Described here is the application of click-chemistry and the use a variety of different glycosylation inhibitors to study and examine the composition of the cell surface glycocalyx of primary cortical neurons. The inhibitors used include kifunensine which is an alkaloid originally isolated from *Kitasatospora kifunense*, an actinobacterium and is a potent inhibitor of the mannosidase I enzyme in the Golgi, but not mannosidase II (Iwami et al., 1987). Use of this inhibitor results in unprocessed oligomannose type glycans, which are not sialylated. Tunicamycin is a glucosamine-containing antibiotic that inhibits the UDP-HexNAc: polyprenol-P family of enzymes and blocks total *N*-linked glycosylation. It inhibits the enzyme GlcNAc phosphotransferase (GPT), which catalyzes the transfer of *N*-acetylglucosamine-1-phosphate from UDP-*N*-acetylglucosamine to dolichol phosphate in the first step of glycoconjugate synthesis, resulting in no *N*-linked glycosylation (Kuo and Lampen, 1974). Myriocin (or ISP-1) is an atypical amino acid and an antibiotic derived from thermophilic fungi including *Mycelia sterilia*. It is a potent inhibitor of serine palmitoyltransferase, an enzyme that catalyzes the first step of sphingolipid biosynthesis and reduces the intracellular pool of sphingolipid intermediates (Kozutsumi et al., 1995). Myriocin also has a secondary role as an immunosuppressant as it suppresses the proliferation of mixed populations of mouse lymphocytes (Fujita et al., 1994). Benzyl 2-acetamido-2-deoxy- α -D-galactopyranoside (α -OBn GalNAc) (*O*-glycosylation inhibitor) inhibits glycosyltransferase incorporation of glucosamine into *O*-glycans. It is a structural analogue of GalNAc- α -1-*O*-serine/threonine and acts as a competitive inhibitor of *O*-glycan chain extension. It has been shown to inhibit mucin *O*-glycosylation in KATO II gastric cancer cells and HM7 colon cancer cells highlighting its potential as an anti-cancer drug (Byrd et al., 1995, Huang et al., 1992). DL-*threo*-PDMP hydrochloride (PDMP) closely resembles the natural sphingolipid substrate of glucosyltransferase and acts as a potent and competitive inhibitor of this enzyme and glucosylceramide synthase. In B16 melanoma cells it has been shown to decrease the levels of glucosylceramide and interfere with monoclonal antibody binding (Inokuchi et al., 1989) and inhibits neurite outgrowth, elongation and branching in SH-SY5Y cells (Hynds et al., 2002).

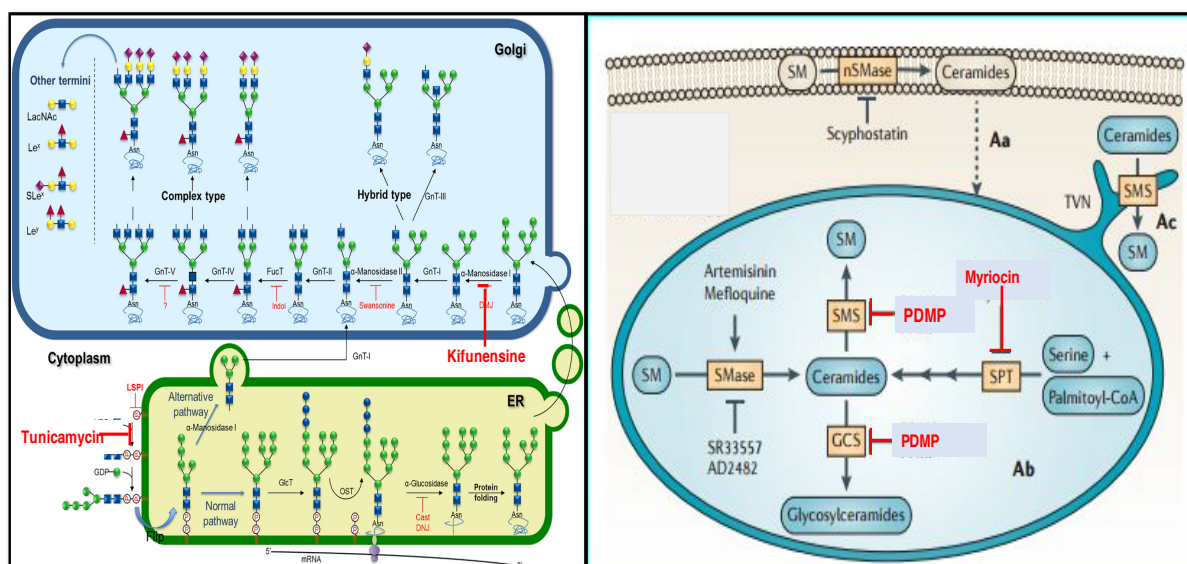


Figure 6.4 – Diagram showing the targets of Kifunensine, Tunicamycin, PDMP and Myriocin inhibition. Kifunenesine inhibits mannosidase I in the Golgi. Tunicamycin inhibits UDP-HexNAc: polyprenol-P, blocking *N*-linked glycosylation. PDMP inhibits glucosyltransferase and Myriocin blocks sphingolipid biosynthesis by inhibiting serine palmitoyltransferase. nSMase – neutral sphingomyelinase; SPT – serine palmitoyltransferase; SM – sphingomyelin; SMS – sphingomyelin synthase; GCS – glucosylceramide synthase. Adapted from Vasconcelos-dos-Santos and Oliveira (2015) and Coppens (2013).

6.1.4 Aims of the chapter

- The application of click-chemistry to study glycosylation and the glycocalyx composition in primary cortical neurons.
- To optimize the labelling of the cell surface/glycocalyx of primary cortical neurons with three synthetic sugars; GlcNAz, GalNAz and ManNAz
- To study the glycocalyx and identify unique glycan structures on the cell surface by use of super resolution (STED) microscopy
- To study the composition of the cell surface glycocalyx of primary cortical neurons through inhibition of different forms of glycosylation using specific inhibitors of *N*-linked, *O*-linked and lipid linked glycosylation

6.2 Methods

6.2.1 Growth of Primary Neuron Cultures

Primary Cortical Neurons were isolated from the brains of one day old neonatal Wistar rats. Rats were decapitated using surgical scissors and the cortices carefully extracted using fine-tipped curved forceps. The tissue was cross chopped and incubated for 25 minutes in 0.3mg/ml trypsin/PBS, at 37 °C. The trypsinisation process was halted by the addition of increasingly concentrated solutions of trypsin inhibitor; PBS containing 0.03mg/ml soybean trypsin inhibitor, 0.06ml/ml, DNase and 3mM MgSO₄, followed by PBS containing 0.1mg/ml soybean trypsin inhibitor, 0.2mg/ml DNase and 10mM MgSO₄. The cortical suspension was re-suspended and passed through a 40µm nylon mesh strainer and the filtrates were centrifuged at 2000 x g for 3 minutes at room temperature. The resultant pellets were re-suspended in 1.5ml Neurobasal medium-A (NBMA, Invitrogen), supplemented with 10% horse serum (HS), 2mM L-Glutamine, penicillin-streptomycin solution (100 units/ml penicillin G, 0.1mg/ml streptomycin sulfate) and 2% B27 and counted, using trypan blue as a diluent. Each brain yielded approximately 3-5 x 10⁶ cells. Immediately after isolation, cells were plated in aforementioned medium, on 13mm glass borosilicate coverslips at a cell density equivalent to the total extract from one cortex per 24 well plate. Each coverslip/dish was coated in 40µg/ml poly-d-lysine (MW 70-150kDa) for 2 hours prior to seeding of cells. After three hours, medium was replaced with NBMA supplemented with 1% HS (all other supplements identical). After 24h, cells were exposed to 20µM Ara-C in 1% HS NBMA to discourage the proliferation of glial cells. After a further 24 hours, Ara-C was removed and cells maintained in Ara-C-free NBMA (0% HS), with 50% medium changes every 3-4 days. Cells were used for experiments at DIV 8-11.

6.2.2 Incorporation of Modified Sugars into the Neuronal Glycome

Synthetically modified azide sugars GlcNAz, GalNAz and ManNAz were produced in the lab of Dr. Eoin Scanlan, Trinity Biomedical Sciences Institute Dublin. Each sugar was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM, aliquoted and stored at -20°C until ready for use. Neurons were treated with 100 μ M of each sugar for 24 hours in NMBA with usual supplements.

6.2.3 Click Chemistry Reaction

Click-chemistry is used to join two compounds together by way of a copper-catalysed or copper free reaction between two functional groups, an azide and an alkyne. The modified sugars were incorporated into the glycan synthesis pathways and incorporated into the glycocalyx and cell surface glycome. This surface azide was then detected upon the addition of a fluorescently labelled alkyne, in the presence of copper.

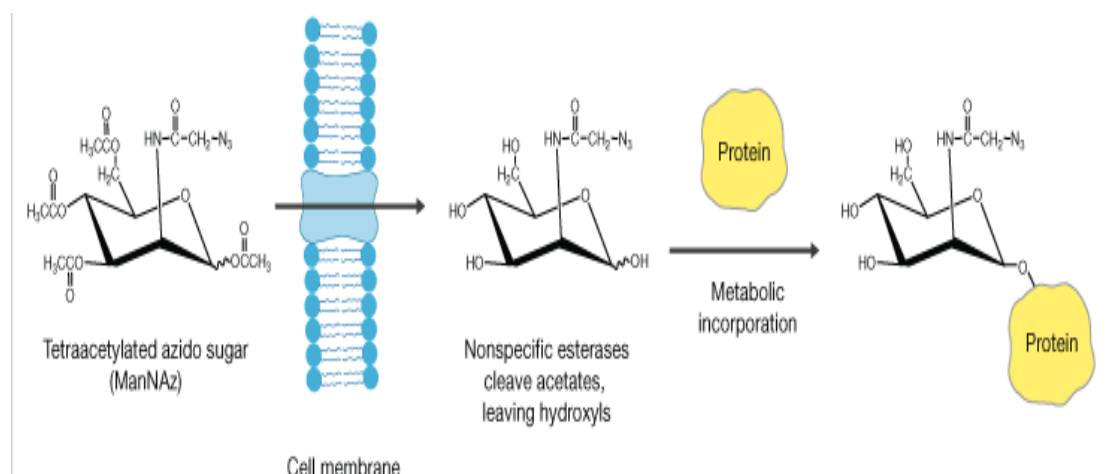


Figure 6.5 – Incorporation of the Click IT reactive, modified sugar ManNAz into the CMP-*N*-acetylmannosamine metabolic pathways for detection on the cell surface. Tetra acetylated ManNAz is membrane permeable and diffuses through the membrane, entering the sialic acid processing pathway. It is incorporated onto surface (and intracellular) sialic acid glycans and detected upon the addition of a fluorescent probe in the presence of copper.

Click reactions were carried out on azido sugar incorporated cells by incubating coverslips in a click reaction containing 25 mM alkyne fluorophore, 50 μ M copper sulphate, 2.5 mM ascorbic acid and 250 μ M THPTA for 5 min. The solution was removed and coverslips were washed five times using warm PBS before cells were fixed using 4% PFA for 15 min. Coverslips were then washed a further three times with PBS and mounted on clear glass slides using either ProLong Anti-Fade or hydromount as a mountant and sealed using clear nail varnish.

6.3 Results

6.3.1 Incorporation of Synthetic Azido Sugars into the Glycocalyx and the Cell Surface Glycome of Primary Cortical Neurons

Primary cortical neurons were incubated with the modified sugars GlcNAz, GalNAz and ManNAz (100 μ M) for 24 hours, 8 days after being isolated from rat pups and grown on poly-D-lysine coated glass coverslips. Cells were washed with PBS and a copper Click IT reaction solution was added containing an Alexa 488 fluorophore catalysing a bond formation between the azide on the modified sugar and the alkyne fluorophore. Following fixation, cells were imaged using a Leica SP8 confocal microscope and the glycocalyx containing the synthetic modified sugars on the neuronal cell surface were visualised. As seen in Fig 6.6, GlcNAz is well incorporated into the cell surface glycome and appears to be concentrated around the surface of the cell body with strong staining on larger neuronal projections. GalNAz shows strong staining around the cell body with a more ubiquitous incorporation throughout neuronal projections (Fig 6.7). Finally, ManNAz shows the most intense staining throughout the cell surface indicating a high concentration of sialic acids in the glycocalyx and the neuronal cell surface glycome as seen in Fig 6.8.

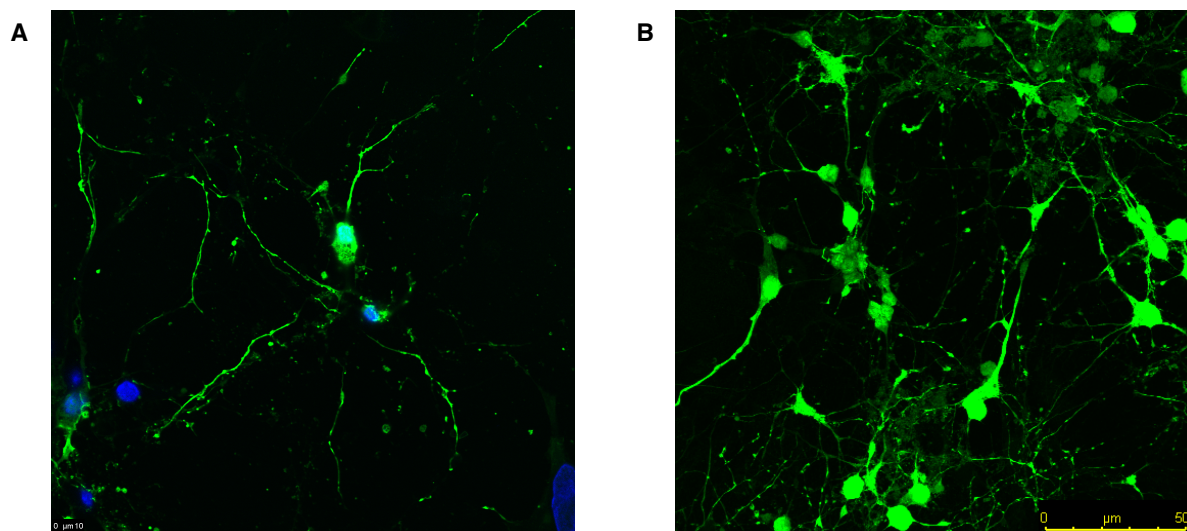


Figure 6.6 – Incorporation of GlcNAz into the cell surface glycome of primary cortical neurons.

Primary cortical neurons were incubated with 100 μ M GalNAz for 24 hrs before being washed in warm PBS 3 times. Then a copper solution containing an Alexa 488 fluorophore was added for 5 min before cells were washed 3 times using warm PBS, fixed using 4% paraformaldehyde and cover slips were mounted on clear glass slides using pro-long Gold with DAPI co-stain (A) or hydromount (B). After being sealed using clear nail varnish, slides were imaged on a Leica SP8 confocal microscope using a 60X objective lens at a resolution of 2048x2048. Image representative of n=3.

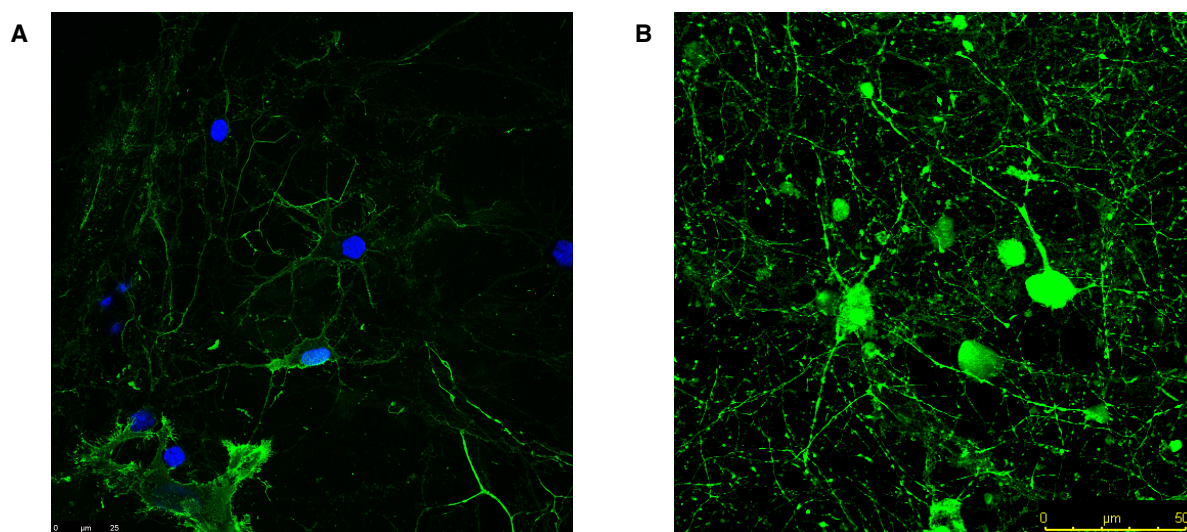


Figure 6.7 – Incorporation of GalNAz into the cell surface glycome of primary cortical neurons.

Primary cortical neurons were incubated with 100 μ M GalNAz for 24 hrs before being washed in warm PBS 3 times. Then a copper solution containing an Alexa 488 fluorophore was added for 5 min before cells were washed 3 times using warm PBS, fixed using 4% paraformaldehyde and cover slips were mounted on clear glass slides using pro-long Gold with DAPI co-stain (A) or hydromount (B). After being sealed using clear nail varnish, slides were imaged on a Leica SP8 confocal microscope using a 60X objective lens at a resolution of 2048x2048. Image representative of n=3.

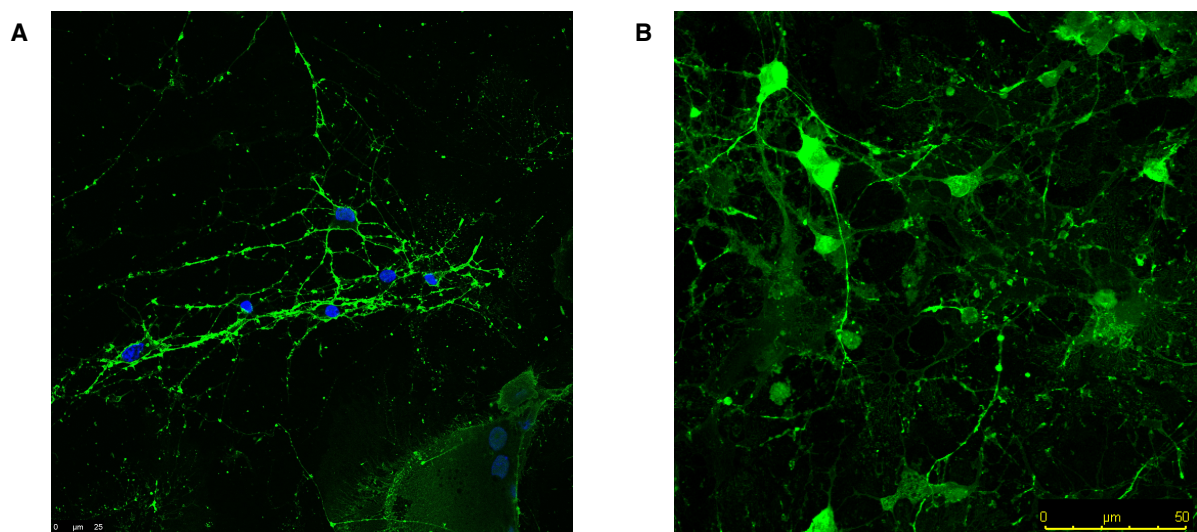


Figure 6.8 – Incorporation of ManNAz into the cell surface glycome of primary cortical neurons.

Primary cortical neurons were incubated with 100 μ M ManNAz for 24 hrs before being washed in warm PBS 3 times. Then a copper solution containing an Alexa 488 fluorophore was added for 5 min before cells were washed 3 times using warm PBS, fixed using 4% paraformaldehyde and cover slips were mounted on clear glass slides using pro-long Gold with DAPI co-stain (A) or hydromount (B). After being sealed using clear nail varnish, slides were imaged on a Leica SP8 confocal microscope using a 60X objective lens at a resolution of 2048x2048. Image representative of n=3.

6.3.2 Differentiation of Neurons does not Influence the Levels of Sialic Acids in the Cell Surface Glycome

Following confirmation of ManNAz incorporation into the neuronal cell surface glycome, the levels of sialic acids on the cell surface were investigated and were found to be consistent throughout the stages of neuronal differentiation, from 96 hours onwards (Fig 6.9). Interestingly, ManNAz incorporation and sialic acid levels were lower compared to GlcNAz and GalNAz around the cell soma when the neurons are fully differentiated, which may indicate a role for this sialylation in axon/dendrite outgrowth or synaptic transmission.

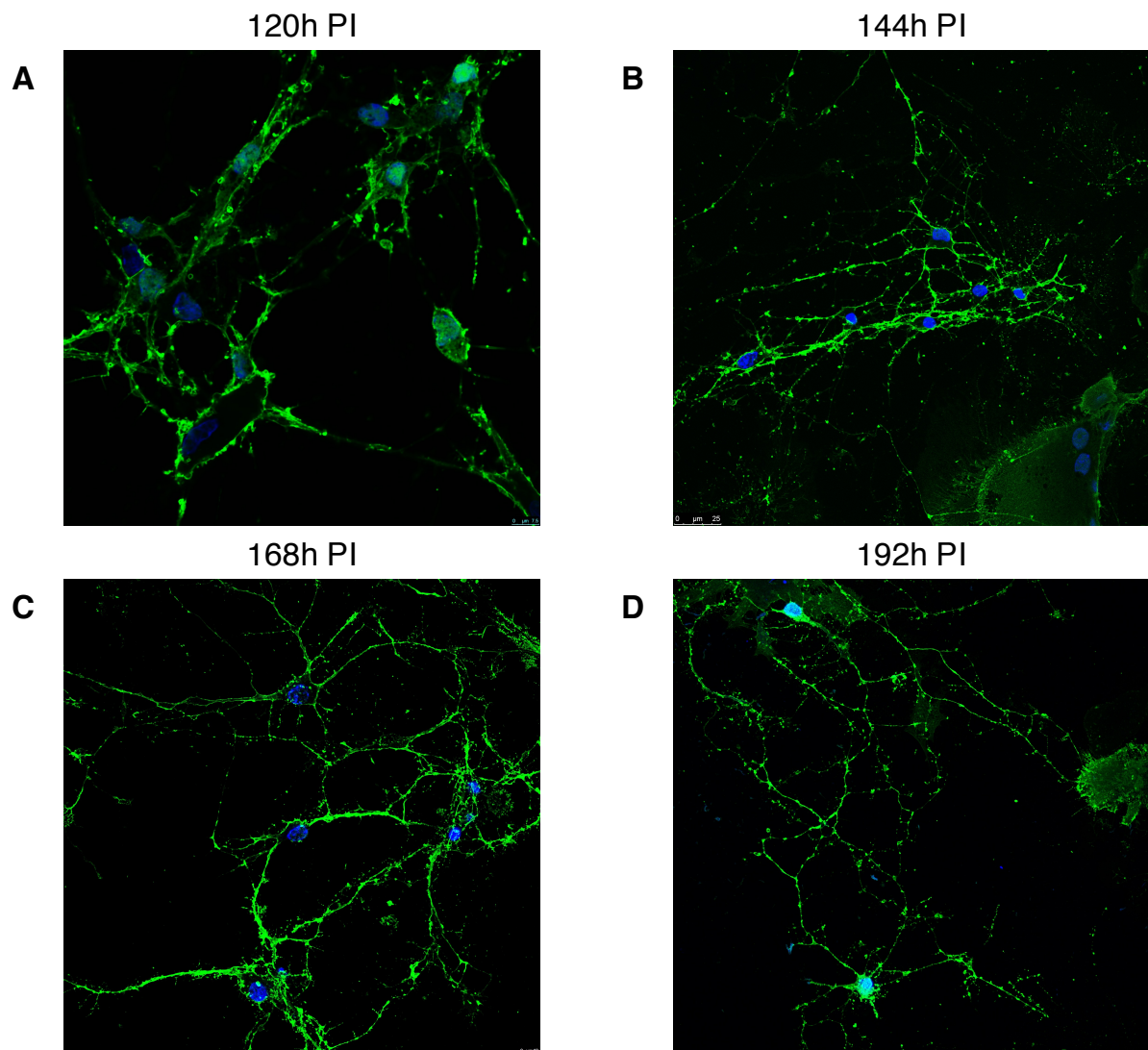


Figure 6.9 – ManNAz is incorporated into the neuronal cell surface glycome in the same manner at later stage of neuronal differentiation. Primary cortical neurons were isolated and grown on poly-D-lysine coated coverslips for 96 hrs (A), 120 hrs (B), 144 hrs (C) and 168 hrs (D) before being incubated with ManNAz for 24 hrs. At this point the copper click IT reaction was initiated by the addition of a solution containing copper and an Alexa 488 fluorophore, cells were washed 3 times in warm PBS, fixed using 4% paraformaldehyde and cover slips were mounted on clear glass slides using pro-long Gold with DAPI co-stain (A). After being sealed using clear nail varnish, slides were imaged on a Leica SP8 confocal microscope using a 60X objective lens at a resolution of 2048x2048. Image representative of n=3. There is no significant difference in fluorescence intensity or staining pattern at the various stages of differentiation. (PI – Post Isolation).

6.3.3 Sialic Acids are not Concentrated at Synaptic Sites

To investigate a potential role for sialic acids in synaptic transmission, fully differentiated neurons were co-stained with synaptophysin, a protein involved in synaptic vesicle release and recycling. Cells were incubated with ManNAz and imaged with an Alexa 594 fluorophore. Following fixation, cells were permeabilized using 0.1% Triton X-100 and incubated with an antibody against synaptophysin overnight. The following day cells were washed and incubated with a secondary antibody conjugated with an Alexa 488 fluorophore allowing us to investigate any co-localization between sialic acids (red) and synaptophysin (green) as seen in Fig 6.10. ManNAz was incorporated ubiquitously throughout the cell but sialic acids do not appear to be concentrated around the sites of synaptic transmission that sialylation is not important for maintaining synaptic connections.

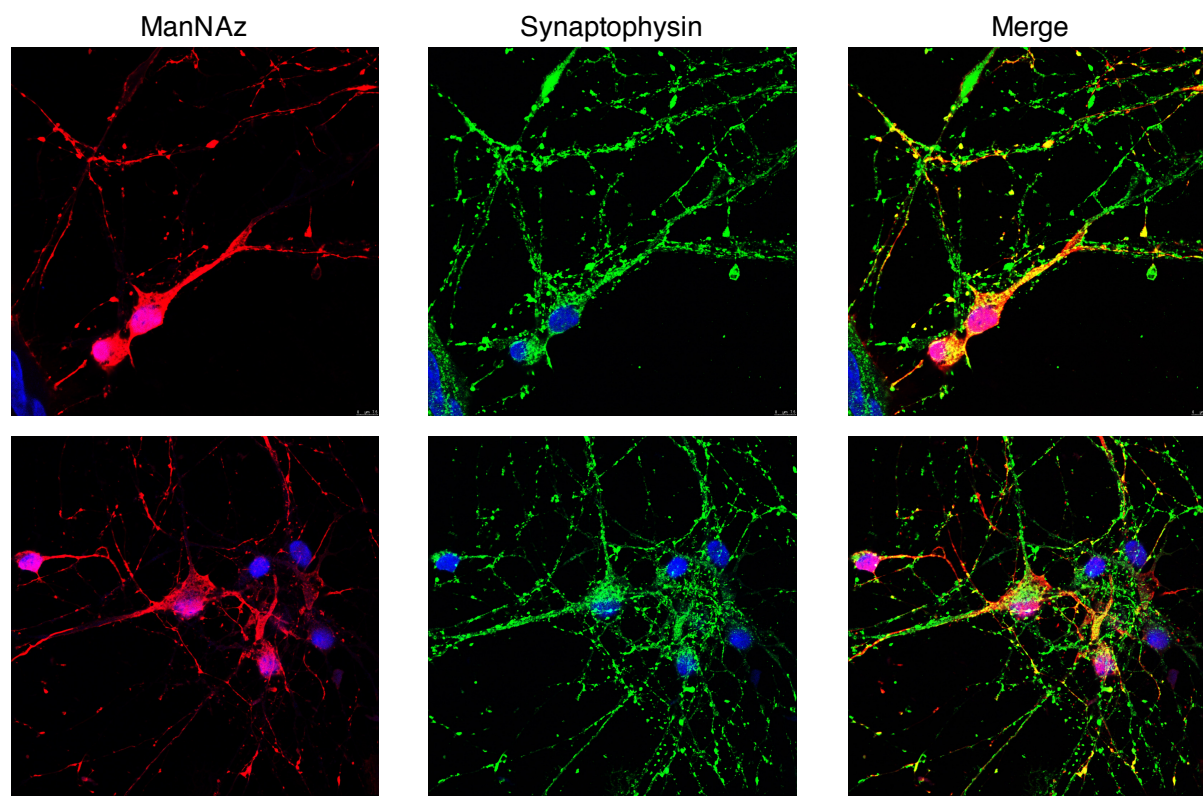


Figure 6.10 – Sialic acids are not concentrated at synaptic sites. Primary cortical neurons were isolated and grown on poly-D-lysine coated coverslips for 8 days before being incubated with 100 μ M ManNAz for 24 hrs. At this point the copper click IT reaction was initiated by the addition of a solution containing copper and an Alexa 567 fluorophore, cells were washed 3 times in warm PBS, fixed using 4% paraformaldehyde. Cells were then permeabilized using 0.1% Triton X-100 and incubated with a primary antibody against synaptophysin overnight at 4 °C in blocking buffer. The next day cells were washed with PBS and incubated with a secondary antibody conjugated with an Alexa 488 fluorophore for 1 hour before cover slips were mounted on clear glass slides using pro-long Gold with DAPI co-stain. After being sealed using clear nail varnish, slides were imaged on a Leica SP8 confocal microscope using a 60X objective lens at a resolution of 2048 x 2048 pixels. Image representative of n=3.

6.3.4 Super Resolution Microscopy of the Cell Surface Glycome of Primary Cortical Neurons

To investigate glycosylated structures on the cell surface super resolution microscopy was performed. Initially, hyvolution was used, a technique which merges optical super-resolution and computational super-resolution using a standard confocal microscope which can provide lateral resolution of up to 140 nm (Borlinghaus and Kappel, 2016). Using this technique, it was possible to observe more detail on the localization of the various sugars but in greater structural detail (Fig 6.11). Similarly, to previous experiments, ManNAz provided the greatest clarity of the neuronal glycolyx and localization of sialic acids on the cell surface. Due to the requirement for a strong fluorescent signal for super-resolution microscopy, ManNAz incorporation into the cell surface glycome with stimulated emission depletion (STED) microscopy was used. STED works by selectively deactivating fluorophores, minimising the area of illumination at the focal point and enhancing the achievable resolution (Westphal et al., 2008). Shown in Fig 6.12 is a whole cell image taken using STED microscopy showing a much greater level of detail than had been seen with other microscopy techniques. By increasing the zoom on a 100X objective lens, it was possible to see individual structures on the neuronal projections of the cell. Most evident were larger “rafts” which possibly contain a number of receptors and other concentrated areas of sialic acids which may be important for neuron branching, receptor localization or synaptic signalling (Fig 6.13). This experiment, shows for the first time the level of detail which STED microscopy can provide of the neuronal cell surface glycome using click-chemistry and synthetic azido sugars. Further work is required to elucidate the structures which present these sialic acids and possibly the function of this carbohydrate structure in neuronal function.

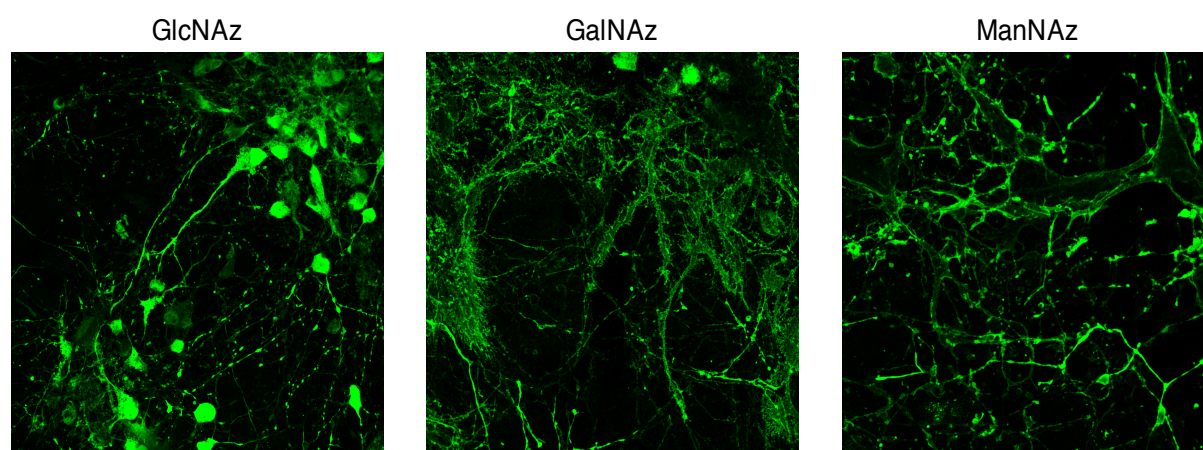


Figure 6.11 – Hyvolution high resolution images of three modified azido sugars showing finer detail of the cell surface glycome of primary cortical neurons. Primary cortical neurons were isolated and grown on poly-D-lysine coated coverslips for 8 days before being incubated with 100 μ M GlcNAz, GalNAz or ManNAz for 24 hrs. At this point the copper click IT reaction was initiated by the addition of a solution containing copper and an Alexa 567 fluorophore, cells were washed 3 times in warm PBS, fixed using 4% paraformaldehyde before cover slips were mounted on clear glass slides using hydromount. After being sealed using clear nail varnish, slides were imaged on a Leica SP8 confocal microscope using a 60X objective lens at a resolution of 2048x2048. Image representative of n=3.

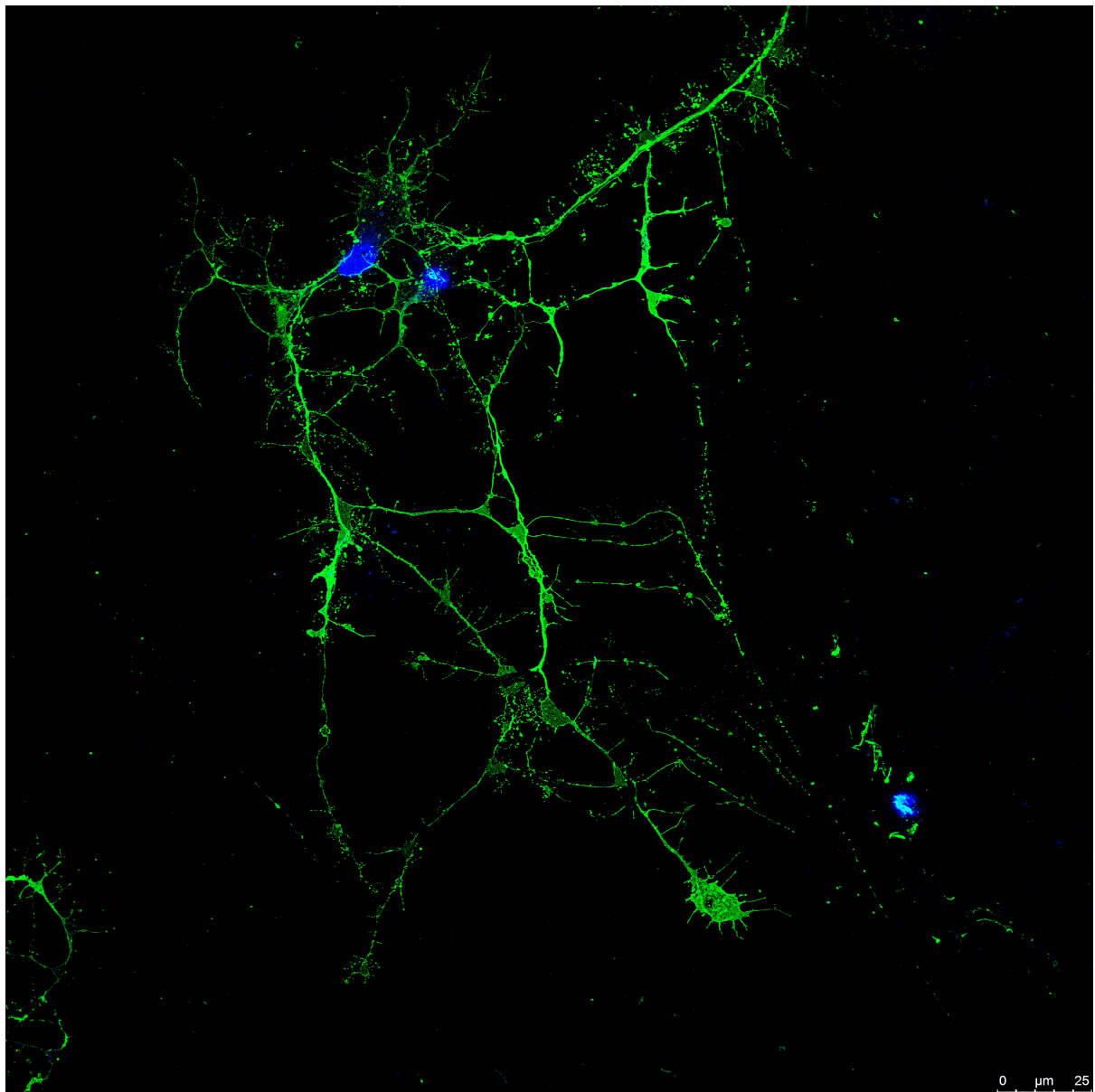


Figure 6.12 – STED image showing incorporation of ManNAz into the cell surface glycome of primary cortical neurons. Primary cortical neurons were incubated with 100 μ M ManNAz for 24 hrs before being washed in warm PBS 3 times. Then a copper solution containing an Alexa 488 fluorophore was added for 5 min before cells were washed 3 times using warm PBS, fixed using 4% paraformaldehyde and cover slips were mounted on clear glass slides using pro-long Gold with DAPI co-stain. After being sealed using clear nail varnish, slides were imaged on a Leica SP8 STED microscope using a 100X objective lens at a resolution of 2048x2048. Image representative of n=3.

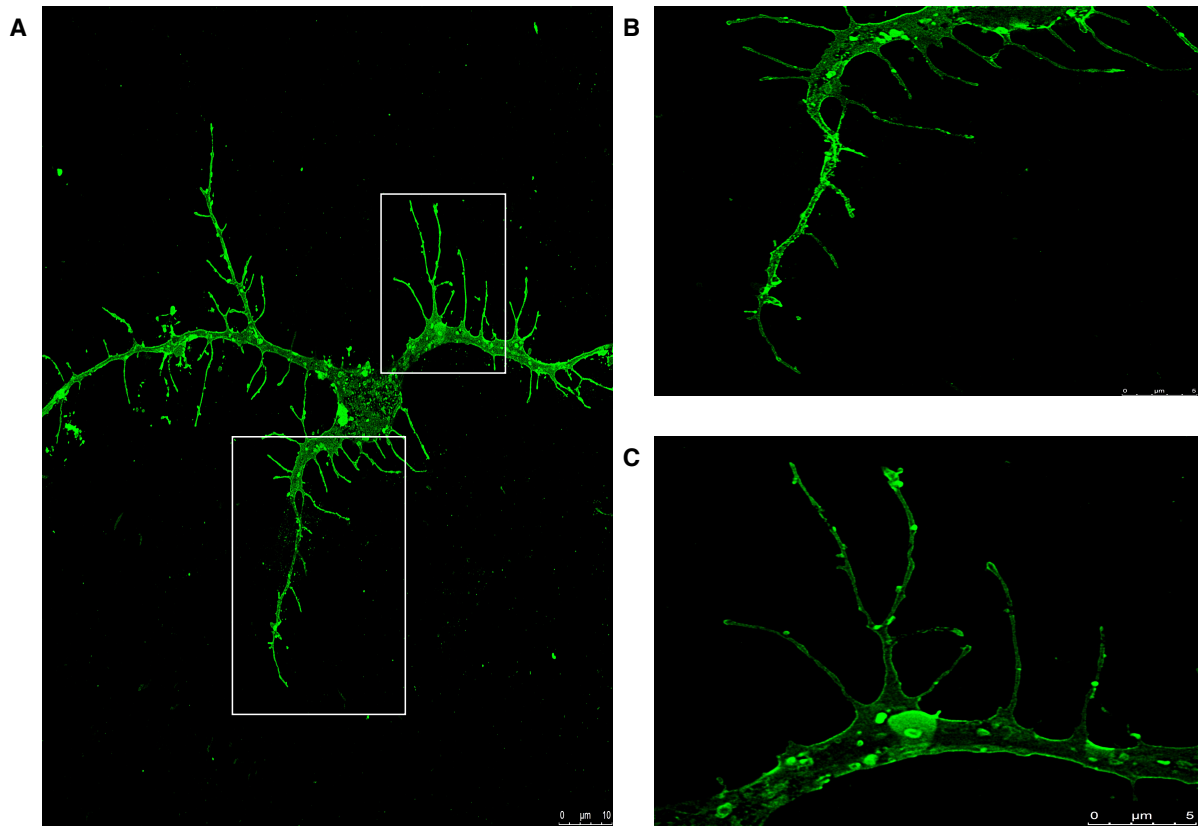


Figure 6.13 – Deconvoluted STED image showing isolated raft and ring glycan structures on the dendritic branches of primary cortical neurons. Primary cortical neurons were incubated with 100 μ M ManNAz for 24 hrs before being washed in warm PBS 3 times. Then a copper solution containing an Alexa 488 fluorophore was added for 5 min before cells were washed 3 times using warm PBS, fixed using 4% paraformaldehyde and cover slips were mounted on clear glass slides using pro-long Gold with DAPI co-stain. After being sealed using clear nail varnish, slides were imaged on a Leica SP8 STED microscope using a 100X objective lens at a resolution of 2048x2048. Image representative of n=3.

6.3.5 Investigation of the Composition of the Neuronal Glycocalyx and Cell Surface Glycome using Click-Chemistry and Inhibition of Glycosylation

Inhibition of glycosylation using glycosyltransferase inhibitors negatively affects the incorporation of the modified azido sugars into the neuronal cell surface glycome and allows the investigation of the composition of the neuronal glycocalyx and cell surface glycome. Primary cortical neurons were incubated with the modified sugars in the presence of glycosyltransferase inhibitors and compared to fluorescence levels of neurons without inhibition to investigate the composition of the cell surface glycome and quantify levels of the different forms of glycosylation. A variety of inhibitors were used including kifunensine; a mannosidase inhibitor (Fig 6.14), tunicamycin; an inhibitor of *N*-linked glycosylation (Fig 6.15), myriocin; an inhibitor of serine palmitoyltransferase and sphingolipid synthesis (Fig 6.16), α -OBn-GalNAc; an inhibitor of *O*-linked glycosylation (Fig 6.17) and PDMP; an inhibitor of brain glucosylceramide synthase (Fig 6.18). It was observed that co-incubation of primary cortical neurons with each inhibitor reduced GlcNAz incorporation into the cell surface glycome. With inhibition and comparison of *N*- and *O*-linked glycosylation it was observed that approximately 60% of the GlcNAz incorporation in these cells is *N*-linked as tunicamycin results in a much greater decrease in fluorescence and pixel intensity (Fig 6.19). GlcNAz is also significantly decreased in cells treated with kifunensine, suggesting a decrease in incorporation into complex type glycans. Reduction of GlcNAz in cells treated with myriocin and PDMP suggests that GlcNAz is also incorporated into sphingolipids and ceramides under control conditions (fig 6.19). A reduction in GalNAz fluorescence was only seen after treatment with tunicamycin and myriocin suggesting that GalNAz is also incorporated into *N*-glycans and sphingolipids under control conditions (Fig 6.20). In cortical neurons, ManNAz fluorescence was only reduced after treatment with kifunensine, indicating the absence of sialic acids and inhibition of complex type glycan formation in the Golgi. Interestingly, there was no decrease in fluorescence after incubation with tunicamycin or OBn-GalNAc, which suggests that sialylation is not reduced by general inhibitors of these forms of glycosylation (Fig 6.21).

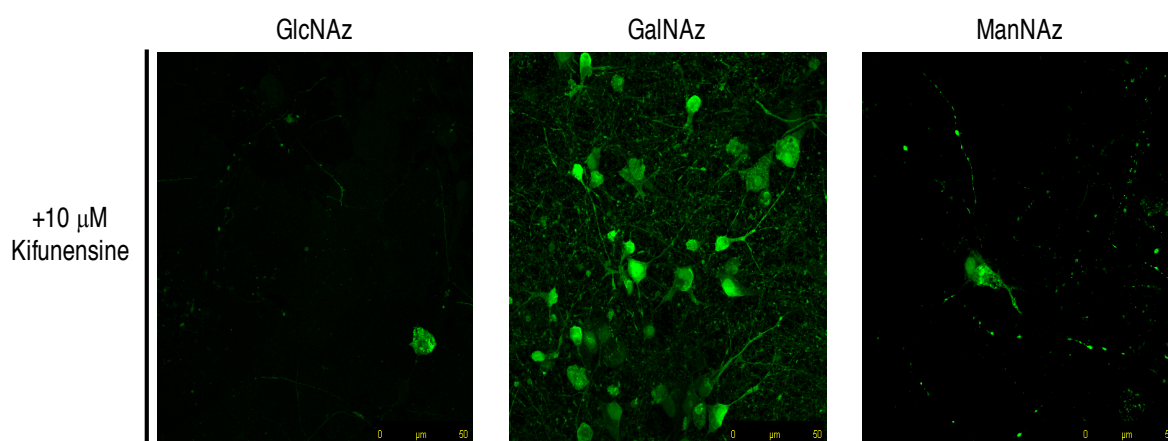


Figure 6.14 – Kifunensine reduces the incorporation of modified azido sugars GlcNAz and ManNAz into the neuronal cell surface glycome. Primary cortical neurons were incubated with 10 μ M kifunensine and 100 μ M GlcNAz, GalNAz or ManNAz for 24 hrs before being washed in warm PBS 3 times. Then a copper solution containing an Alexa 488 fluorophore was added for 5 min before cells were washed 3 times using warm PBS, fixed using 4% paraformaldehyde and cover slips were mounted on clear glass slides using hydromount. After being sealed using clear nail varnish, slides were imaged on a Leica SP8 confocal microscope using a 60X objective lens at a resolution of 2048x2048. Image representative of n=3.

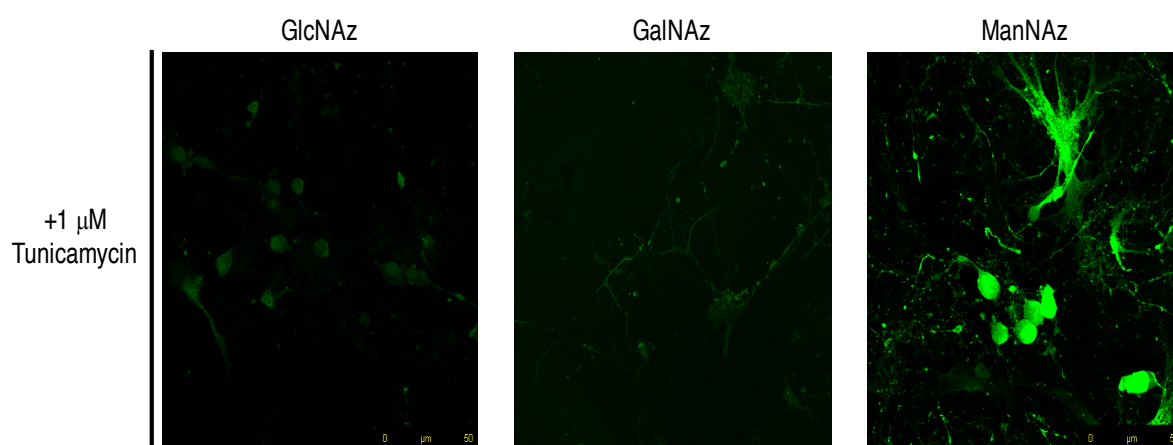


Figure 6.15 – Tunicamycin reduces the incorporation of modified azido sugars GlcNAz and GalNAz into the neuronal cell surface glycome. Primary cortical neurons were incubated with 1 μM tunicamycin and 100 μM GlcNAz, GalNAz or ManNAz for 24 hrs before being washed in warm PBS 3 times. Then a copper solution containing an Alexa 488 fluorophore was added for 5 min before cells were washed 3 times using warm PBS, fixed using 4% paraformaldehyde and cover slips were mounted on clear glass slides using hydromount. After being sealed using clear nail varnish, slides were imaged on a Leica SP8 confocal microscope using a 60X objective lens at a resolution of 2048x2048. Image representative of n=3.

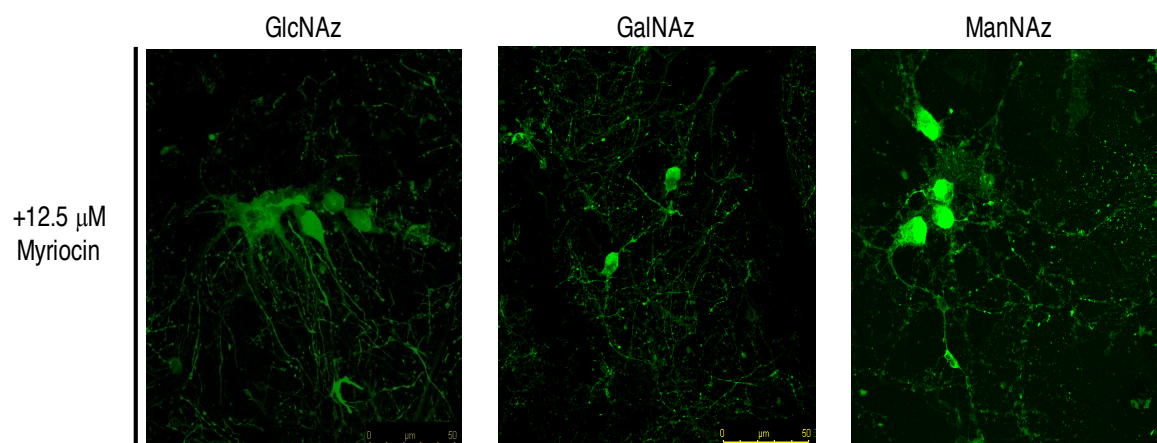


Figure 6.16 – Myriocin reduces the incorporation of modified azido sugars GlcNAz and GalNAz into the neuronal cell surface glycome. Primary cortical neurons were incubated with 12.5 μM myriocin and 100 μM GlcNAz, GalNAz or ManNAz for 24 hrs before being washed in warm PBS 3 times. Then a copper solution containing an Alexa 488 fluorophore was added for 5 min before cells were washed 3 times using warm PBS, fixed using 4% paraformaldehyde and cover slips were mounted on clear glass slides using hydromount. After being sealed using clear nail varnish, slides were imaged on a Leica SP8 confocal microscope using a 60X objective lens at a resolution of 2048x2048. Image representative of n=3.

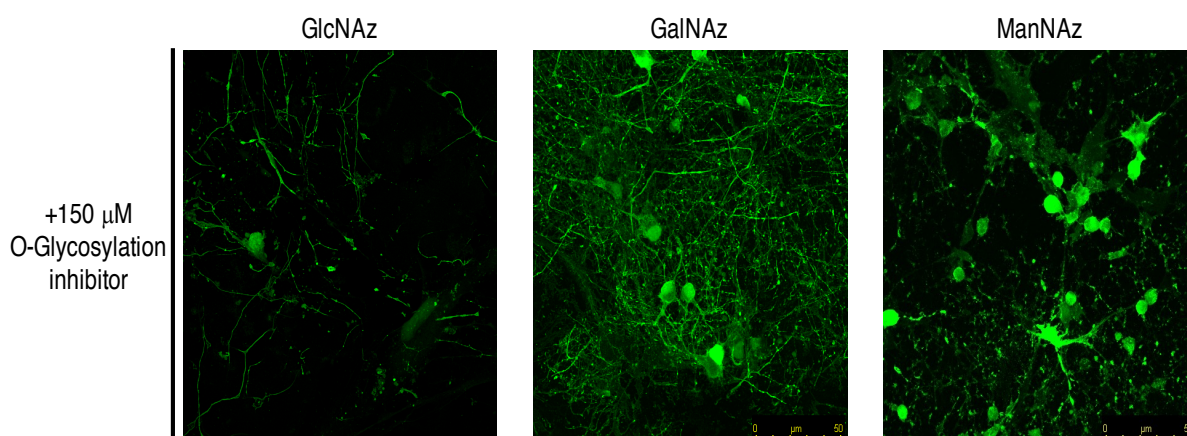


Figure 6.17 – α -OBn GalNAc reduces the incorporation of modified azido sugars GlcNAz into the neuronal cell surface glycome. Primary cortical neurons were incubated with 150 μ M α -OBn GalNAc and 100 μ M GlcNAz, GalNAz or ManNAz for 24 hrs before being washed in warm PBS 3 times. Then a copper solution containing an Alexa 488 fluorophore was added for 5 min before cells were washed 3 times using warm PBS, fixed using 4% paraformaldehyde and cover slips were mounted on clear glass slides using hydromount. After being sealed using clear nail varnish, slides were imaged on a Leica SP8 confocal microscope using a 60X objective lens at a resolution of 2048x2048. Image representative of n=3.

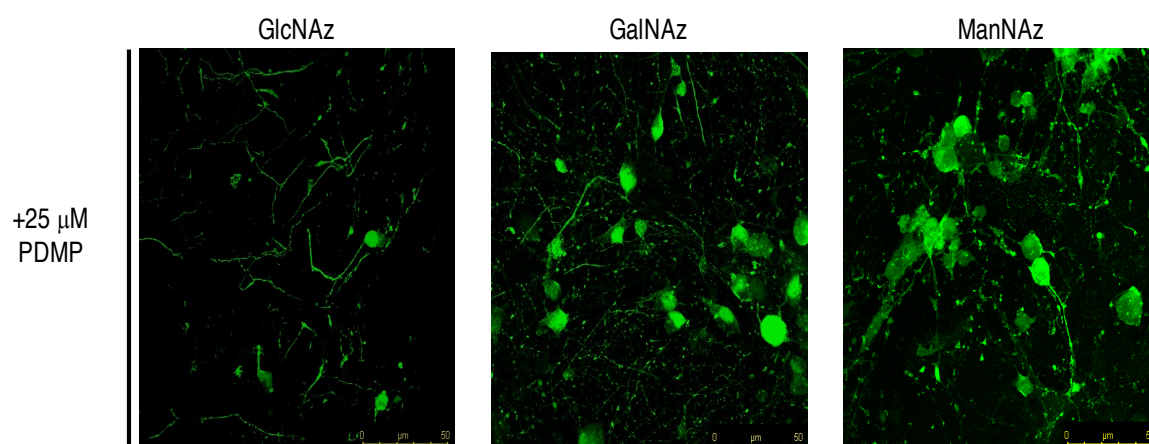


Figure 6.18 – PDMP reduces the incorporation of modified azido sugars GlcNAz into the neuronal cell surface glycome. Primary cortical neurons were incubated with 25 μ M PDMP and 100 μ M GlcNAz, GalNAz or ManNAz for 24 hrs before being washed in warm PBS 3 times. Then a copper solution containing an Alexa 488 fluorophore was added for 5 min before cells were washed 3 times using warm PBS, fixed using 4% paraformaldehyde and cover slips were mounted on clear glass slides using hydromount. After being sealed using clear nail varnish, slides were imaged on a Leica SP8 confocal microscope using a 60X objective lens at a resolution of 2048x2048. Image representative of n=3.

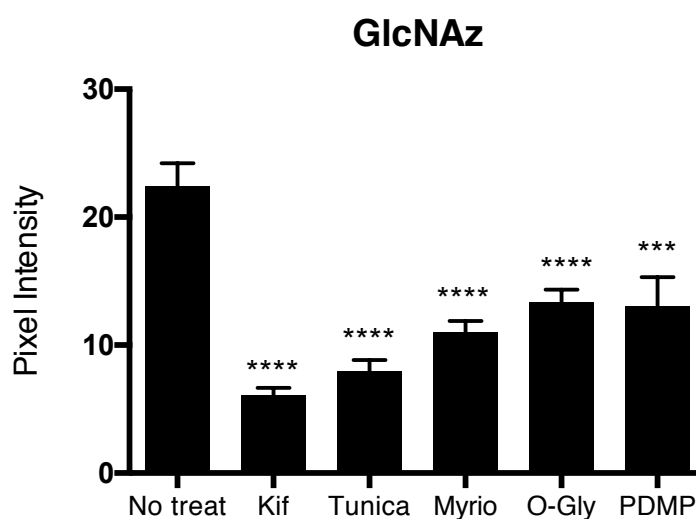


Figure 6.19 – Inhibition of glycosylation reduces the incorporation of GlcNAz into the neuronal cell surface glycome. Images taken using a Leica SP8 confocal microscope were analysed using Leica SP8 confocal suite and ImageJ to get the mean pixel intensity of any green fluorescence for each image minus background noise. Data shows a reduction in pixel intensity indicating a reduction in the incorporation of GlcNAz into the neuronal cell surface glycome as compared to untreated cells. Data compiled from at least 5 different fields of view from three separate slides prepared on different days. Data presented as mean $\pm$ SEM, n=3.

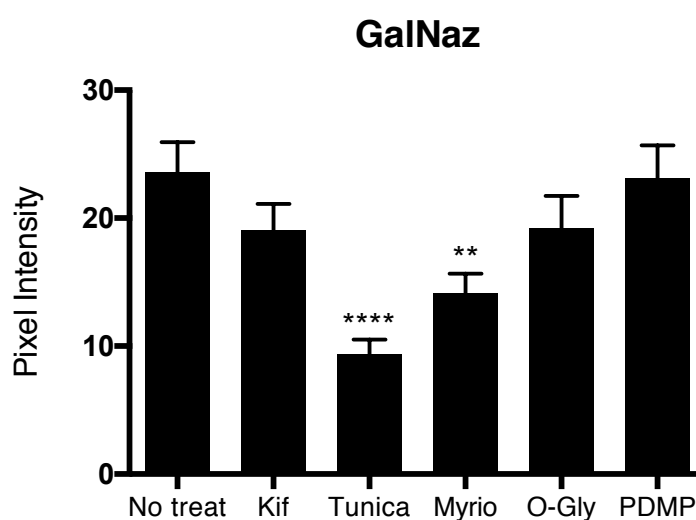


Figure 6.20 – Inhibition of *N*-linked glycosylation and serine palmitoyltransferase reduces the incorporation of GalNAz into the neuronal cell surface glycome. Images taken using a Leica SP8 confocal microscope were analysed using Leica SP8 confocal suite and ImageJ to get the mean pixel intensity of any green fluorescence for each image minus background noise. Data shows a reduction in pixel intensity indicating a reduction in the incorporation of GalNAz into the neuronal cell surface glycome of tunicamycin and myriocin treated cells as compared to untreated cells. Data compiled from at least 5 different fields of view from three separate slides prepared on different days. Data presented as mean $\pm$ SEM, $n=3$.

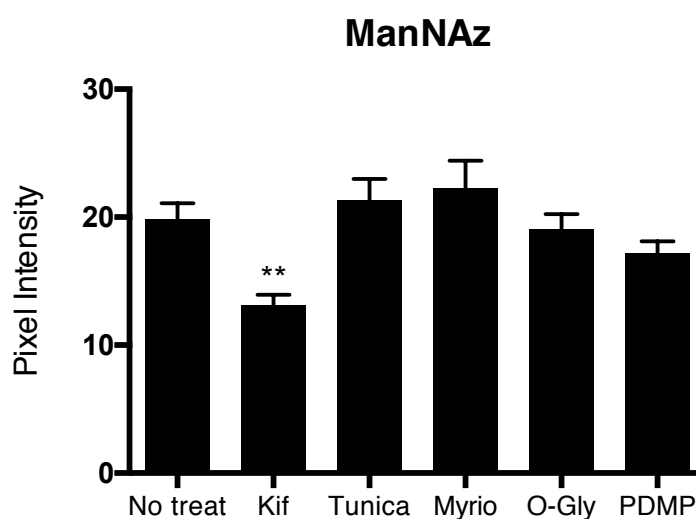


Figure 6.21 – Inhibition of mannosidase I reduces the incorporation of ManNAz into the neuronal cell surface glycome. Images taken using a Leica SP8 confocal microscope were analysed using Leica SP8 confocal suite and ImageJ to get the mean pixel intensity of any green fluorescence for each image minus background noise. Data shows a reduction in pixel intensity indicating a reduction in the incorporation of ManNAz into the neuronal cell surface glycome of kifunensine treated cells as compared to untreated cells. Data compiled from at least 5 different fields of view from three separate slides prepared on different days. Data presented as mean $\pm$ SEM, n=3.

Sugar	Inhibitor	Inhibits	Reduces sugar incorporation (✓)
GlcNAz	kifunensine	mannosidase I	✓
GalNAz			
ManNAz			✓
GlcNAz	tunicamycin	<i>N</i> -linked glycosylation	✓
GalNAz			✓
ManNAz			
GlcNAz	myriocin	serine palmitoyltransferase	✓
GalNAz			✓
ManNAz			
GlcNAz	α -OBn GalNAc	<i>O</i> -linked glycosylation	✓
GalNAz			
ManNAz			
GlcNAz	PDMP	Glucosylceramide synthase	✓
GalNAz			
ManNAz			

Fig 6.22 – Table cataloguing the effects of a variety of inhibitors of glycosylation on the incorporation of modified azido sugars GlcNAz, GalNAz and ManNAz into the neuronal cell surface glycome. All inhibitors reduced the incorporation of GlcNAz in the cell surface glycome while only kifunensine reduced incorporation of ManNAz. GalNAz incorporation was inhibited by tunicamycin and myriocin.

6.4 Discussion

Glycans play key roles in numerous biological processes, particularly on the cell surface, where they are involved in cell-cell interactions such as embryonic development, leukocyte homing and cancer cell metastasis. They are enzymatically attached to a variety of different substrates to make glycoproteins, glycolipids and proteoglycans adding structural diversity that exceeds any linear biopolymers. Glycans are not directly encoded by the mammalian genome and so it is therefore difficult to predict the glycome expressed within and on a given cell type, even with the use of advanced computer models. As a result, glycans have become an attractive target for imaging and techniques such as bio-orthogonal click-chemistry have been developed to study the composition of the glycocalyx and the cell surface glycome. Herein, methods for metabolic labelling using synthetic modified monosaccharides to visualize the cell surface glycome of primary cortical neurons are described.

Most glycans have been imaged using metabolic labelling with azido or alkynyl monosaccharide forms of ManNAc (Saxon and Bertozzi, 2000), GalNAc (Hang et al., 2003), GlcNAc (Vocadlo et al., 2003) and fucose (Rabuka et al., 2006). These chemical reporters have been used to study a wide variety of cultured cells as well as *in vivo* in living organisms such as zebrafish (Laughlin et al., 2008) and in living mice and brain sections (Xie et al., 2016). Despite the association of dysfunctional glycosylation with neurological disorders and the importance of cell surface glycosylation in neuronal outgrowth, little is known about the composition of the cell surface glycome. Furthermore, glycosylation appears to be especially important in maintenance and correct function of ion channels, yet little is known about the types of glycans involved in this function. It has been shown that deglycosylated voltage gated potassium (K^+) channels have slower activation and deactivation rates in neuroblastoma cells (Hall et al., 2011). Voltage gated sodium (Na^+) channels have also been shown to be heavily glycosylated in mammals comprising up to 30% of the total molecular mass of the channel. Interestingly, sialic acids can represent up to 50% of these glycans chains which is important due to the negative charge and potential electrostatic effect of these sugars (Scott and Panin, 2014a). Similarly to K^+ channels,

reduction of sialylation can significantly reduce the electrical conductance properties of Na⁺ channels, indicating the importance of these PTMs in fast neuronal transmission (Cronin et al., 2005). Many ligand gated ion channels have also been shown to be glycosylated and nicotinic acetylcholine receptors (nAChRs) possess several sites where these carbohydrate modifications are important for surface expression, agonist dependent gating and desensitization (Corrie et al., 2005).

Herein, we describe metabolic labelling using click-chemistry to visualize the cell surface glycome of primary cortical neurons for the first time. One previous study has demonstrated the effectiveness of this technique in labelling polysialic acids in hippocampal neurons (Kang et al., 2015). The study reported in this chapter is novel as it uses a variety of different modified sugars, super resolution microscopy and the use of inhibitors to determine the types of glycosylation and composition of the glycocalyx and cell surface glycome. In the study by Kang et al., 2015, it was found that incubating cells in culture with ManNAz resulted in a large decrease in cell viability and so incubation with the sugar occurred directly after isolation and before dissociation of the neurons to limit any toxic effects. Here, we observed no toxic effects with any of the modified sugars when cells were treated 8 days post isolation or when they were treated at various time points during differentiation. Effects on differentiation and viability at the earliest time points (48 hours post isolation) are possible as the click reaction was ineffective and neuronal differentiation appeared to be at varying stages but this was not investigated further. In addition, a longer 48 hour treatment with 50 μ M GlcNAz, GalNAz or ManNAz had no toxic effect or reduction in the incorporation of the modified sugar. It is possible that the toxic effects observed by Kang et al., 2015 are specific to isolated hippocampal neurons which may rely more on polysialic acids for neuronal differentiation or perhaps more likely, incubation of ManNAz at early time points in differentiation has toxic effects but this is reduced 72 hours post isolation. Results shown here describe the successful incorporation of GlcNAz, GalNAz and ManNAz into the cell surface glycome of fully differentiated primary cortical neurons (Fig 6.6, 6.7 and 6.8). Interestingly, the incorporation of GlcNAz and GalNAz occurs mostly around the cell soma indicating this area of the cell has a higher concentration of glycoconjugates containing these monosaccharides,

including *N*-linked and *O*-linked glycoproteins. In contrast, ManNAz is more concentrated along neuronal projections suggesting the axons and dendrites of these cells express high levels of sialic acids.

Kang et al. (2015) found a decrease in the fluorescence intensity of primary hippocampal neurons treated with ManNAz over the course of a 10 day differentiation post isolation. New isolated neurons imaged 1 day *in vitro* (DIV) showed strong fluorescence staining around the cell soma but the intensity decreased gradually with imaging at 3, 7 and 10 DIV. Fig 6.9 shows there was no change in fluorescence intensity over the course of differentiation in primary cortical neurons. A potential reason for this is that cortical neurons which use a variety of neurotransmitters require greater levels of cell surface sialylation for correct function than the cholinergic neurons of the hippocampus. The high level of fluorescence seen on neuronal projections in cortical neurons agrees with the hypothesis that voltage gated ion channels in this type of neuron are highly sialylated. There may also be subtle differences in the application of the technique but it appears unlikely that this would result in the large differences seen between our study and that of Kang. It would be interesting to examine the cell surface sialylation of cortical neurons at the earliest possible time-point (48 hours post isolation) to check for any toxicity and if levels of sialylation are maintained throughout early stages of differentiation.

STED microscopy is a recently developed technique that can give lateral resolution of 70-90 nm, providing much greater detail of cellular structures than is possible with traditional confocal microscopy. One previous study by Letschert et al. (2014) has utilised metabolic labelling to examine the cell surface glycome of osteosarcoma cells using super-resolution microscopy (dSTORM) to show simple structures on the plasma membrane with the greatest density observed with GalNAz containing structures. Using STED microscopy we observe unknown glycan containing structures on dendritic branches of primary cortical neurons (Fig 6.13). Specifically, we observe an unknown circular structure in the middle of a sialylated “raft”. Shown in Fig 6.9 is limited co-localization between sialic acids and synaptic sites and it therefore appears unlikely that the structures identified by STED microscopy are part of the synaptic

machinery. Alternatively, the circular structures suggest that this may be a component of a voltage/ligand gated ion channel which is heavily sialylated on the extracellular side. This would represent the first time the sialylation pattern of an individual channel had been identified by fluorescence microscopy. Previously, super resolution microscopy has been used to image single voltage gated Na⁺ channels in PC12 cells using labelled saxitoxin, a shellfish poison which binds to the extracellular pore of a subset of Na⁺ channels (Ondrus et al., 2012). To investigate these unknown sialylated neuronal structures further co-staining of Na⁺ channels using saxitoxin or a ligand of K⁺ channels, with ManNAz could be used to confirm if the circular structures represent the extracellular sialylation patterns of ion channels.

A variety of glycosylation inhibitors were used to investigate the composition of the neuronal cell surface glycome and the various types of glycans present. Results of these experiments indicate the composition of the glycocalyx of neurons can be determined using this technique and can provide insight into the role of glycans in neuronal differentiation and function. None of the inhibitors appeared to be toxic to the neurons despite the 24 h incubation time. This result suggests that no single type of glycosylation is vital for cellular viability in primary cortical neurons and is surprising considering the wide variety of neuronal processes that glycosylation is involved in including neurotransmitter synthesis, neurotransmitter release, receptors, voltage gated ion channels and transient receptor potential channels (Khidekel et al., 2007, Scott and Panin, 2014b). Further experiments are required to investigate the effects of the inhibitors on different stages of neuronal differentiation and the cell surface glycome at each stage of differentiation. In addition, future work should investigate the role of glycosylation in neurotransmitter release and neuronal cell membrane potential to see how important glycosylation is for function of primary cortical neurons in their fully differentiated state.

Each inhibitor resulted in a significant reduction in the incorporation of at least one of the modified sugars used in the experiments. *N*-glycosylation is the dominant form of protein glycosylation in cortical neurons, as shown by the incorporation of GlcNAz. Interestingly and for unknown reasons only the incorporation of GlcNAz was

decreased by the *O*-glycosylation inhibitor α -OBn GalNAc, which should cause a decrease in GalNAz incorporation and *O*-glycosylation. This result is somewhat surprising as mucin type *O*-linked glycosylation is initiated by the addition of a GalNAc to the hydroxyl group of Serine/threonine side chains (Hanisch, 2001), yet no reduction was seen in GalNAz incorporation with use of this inhibitor. There is little evidence in the literature for mucin type glycosylation in the brain and defects associated with this PTM result in dysfunction of the immune or colorectal systems so it is likely that this type of glycosylation does not occur in cortical neurons. Sialic acids are more commonly incorporated into *N*-glycans and glycolipids so it is not surprising that this inhibitor had little effect on ManNAz incorporation into the cell surface glycome.

Kifunensine is a potent inhibitor of mannosidase I in the Golgi and caused a decrease in GlcNAz and ManNAz incorporation into the neuronal cell surface glycome. These results are expected as inhibition of mannosidase I results in formation of oligomannose type glycans (Man₉) in the Golgi which cannot be sialylated and inhibition of complex glycans, resulting in the observed decrease in GlcNAz and ManNAz incorporation and cell surface sialylation. In this situation a buildup of ManNAz occurs in the ER as it cannot be further processed into sialic acids, thus reducing its incorporation into the cell surface glycome (Avezov et al., 2008). Mannosidase I is also involved in trimming of GlcNAc on *N*-linked glycans so this reduction in GlcNAz incorporation was also expected. Tunicamycin is an inhibitor of *N*-linked glycosylation and decreased GlcNAz and GalNAz incorporation into the cell surface glycome, as expected. It would be also expected that tunicamycin would also reduce ManNAz incorporation, however, significant amounts of sialylated glycolipids are observed.

Myriocin inhibits the initial step of sphingolipid synthesis and also reduces the incorporation of GlcNAz and GalNAz into the neuronal surface glycome. Sphingolipids, or glycosylceramides, are fatty acid derivatives of sphingosine and occur most commonly in the cell membranes of the brain and nervous tissue. Ceramides are a type of sphingolipid that are *N*-acetylated which lack additional head groups and are commonly linked to the ceramide backbone via a GlcNAc or GalNAc

residue (Bergfeld et al., 2012). Thus, myriocin inhibition of serine palmitoyltransferase resulting in a reduction in GlcNAz and GalNAz incorporation was expected. Sialic acid containing glycosphingolipids are referred to as gangliosides, which are also most abundant in the brain, however, there was no reduction in ManNAz incorporation after myriocin treatment. Despite the abundance of gangliosides in the brain, the presence of many simple gangliosides appears to be downregulated in mature neurons and they appear to play a more important role in brain development. In addition to this, mice lacking “brain type” gangliosides display normal histogenesis of brain and gross behavior which may imply the roles of gangliosides in the mature brain is not vital and therefore explains the lack of reduction in ManNAz incorporation observed here (Yu et al., 2011). Gangliosides are also present in myelin and due to the lack of oligodendrocytes in the *in vitro* neuronal preparation, this may have reduced the levels of natural gangliosides in these primary cortical neurons (Vyas et al., 2002). PDMP is an inhibitor of glucosyltransferase and as expected, reduces GlcNAz incorporation into the neuronal cell surface glycome. This compound has been shown to reduce glucosphingolipid expression (Kovács et al., 2000) and so as expected reduction in GlcNAz incorporation is observed but no effect on GalNAz or ManNAz.

The results presented here confirm that modified azido sugars can be used to metabolically label primary cortical neurons and that super resolution microscopy of these sugars can provide us with a detailed view of the structural organization of glycoconjugates on the neuronal cell surface and visualization of glycosylated substructures such as voltage/ligand gated ion channels is possible. In addition, the use of glycosylation inhibitors has elucidated some of the composition of the cell surface glycome by examining the incorporation of each sugar in the presence of a variety of inhibitors through fluorescence intensity. GlcNAz appears to be present in both *N*- and *O*-glycans as well as in sphingolipids and glycoproteins and thus is the most predominantly expressed glycan in these neurons. GalNAz is only incorporated into *N*-glycans in the brain but is present in both glycoproteins and sphingolipids and evidenced in the varying decreases in incorporation after tunicamycin and myriocin treatment. ManNAz incorporation and sialylation are the major forms of glycosylation observed in cortical neurons and this is not inhibited by tunicamycin or by inhibition of

O-glycosylation.

Chapter 7

General Discussion

7.1 General Discussion

Mitochondrial dysfunction has been a hallmark of many neurodegenerative diseases for a number of years but in the last decade much of the focus has shifted towards altered mitochondrial dynamics as a potential element for disease onset/progression. This shift in focus has come as a result of increasing evidence that defects in a variety of different aspects of mitochondrial dynamics have been associated with genes already known to be involved in neurodegenerative disease progression. Mitochondrial quality control has been implicated in PD as a result of mutations in PINK1/parkin known to be causative of the disease, an increase in mitochondrial fission as a result of Drp1 activation in AD, defects in the fusion machinery in CMT disease (type 2A) and mitochondrial transport deficits to the synapse which are evident in a variety of neurodegenerative diseases. One common theme which is conspicuous in all cases of neurodegeneration is the loss of synaptic connectivity in distinct brain regions. Mitochondrial dynamics is crucial to this process as it allows for changes in mitochondrial shape and transport to synaptic sites and maintenance of general mitochondrial health through sharing of mtDNA in organelles which are particularly susceptible to oxidative damage. The general aim of this thesis was to link alterations in mitochondrial dynamics, induced by bioenergetics dysfunction or altered glycosylation, to the cellular dysfunction seen in neurodegeneration. Differentiated PC12 cells and primary cortical neurons were used to examine the implications of bioenergetics defects, inhibition of substrate transport and altered O-GlcNAcylation levels on mitochondrial dynamics.

Previous studies have described the effects of ETC inhibition on mitochondrial morphology, $\Delta\psi_m$ and ROS production in a variety of cell types (Barsoum et al., 2006, Kaasik et al., 2007), yet little none have examined the effect of these inhibitors on mitochondrial fusion in neurons or a neuronal model despite the evidence linking dysfunction of the ETC with neurodegeneration. Chapter 3 of this thesis shows that inhibition of the ETC of dissipation of the $\Delta\psi_m$ strongly inhibits mitochondrial fusion in differentiated PC12 cells, with most of the inhibitors

showing close to 100% inhibition of fusion as measured by an assay measuring mitochondrial fusion through the sharing of a photo-activatable green signal amongst mitochondria (Fig 3.4-3.9). This abolition of fusion is accompanied by significant alterations in mitochondrial membrane potential (Fig 3,14), but only CIII inhibition resulted in a significant increase in ROS production (Fig 3.15). Opa1 is a dynamin-like GTPase which controls MIM fusion and exists in two forms; L-Opa1 and S-Opa1 (Ishihara et al., 2006). Under physiological conditions, there exists a balance between these two forms which mediates successful MIM fusion. However, under conditions of cellular stress, such as a loss of $\Delta\psi_m$ or drastic reduction in ATP levels, L-Opa1 is completely cleaved to the short form inhibiting MIM fusion. In Fig 3.17, we show a sharp decrease in the ratio of L-Opa1:S-Opa1 from 2 to below 1.5 and below 1 after 20 minute treatment with FCCP. These results demonstrate that the loss of $\Delta\psi_m$ as a result of ETC inhibition or the loss of ATP production through inhibition of ATP synthase are sufficient to cleave L-Opa1 enough to inhibit mitochondrial fusion in a short time frame. Unpublished data from the lab demonstrates that ATP production is significantly reduced with similar high concentrations of OXPHOS inhibitors indicating L-Opa1 may be cleaved by either Oma1 or Yme1L1 as a result of oxidative stress, but conclusively proves that cleavage of Opa1 is sufficient to block mitochondrial fusion after only 20 mins in differentiated PC12 cells. It should also be noted that the mitochondrial network appears fragmented after treatment with OXPHOS inhibitors and this is unlikely to be as a result of cessation of fusion for 45 mins. Instead, the mitochondrial stress invoked by these inhibitors likely results in an increase in cytosolic calcium, activating Drp1 and increasing its catalytic activity by recruiting it to the mitochondrial membrane (Cribbs and Strack, 2007). This chapter highlights the dual effects of OXPHOS inhibition of inhibition of fusion and activation of fission, which links inhibition of OXPHOS with activation of the cell death pathway in a neuronal model (Breckenridge et al., 2003).

Furthermore, Chapter 3 showed the existence of a CI threshold effect with inhibition of mitochondrial fusion in primary cortical neurons. Incubation of primary cortical neurons with 10 nM or 15 nM piericidin A has no effect on mitochondrial

fusion despite inhibiting complex I by 43% and 46% respectively. Incubation with 17.5 nM piericidin A slightly reduces mitochondrial fusion with inhibition a 53% inhibition of CI. However, 20 nM piericidin A completely inhibits mitochondrial fusion after only 5 mins while complex I is inhibited by 55% confirming that complex I must be inhibited by at least 53% before mitochondrial fusion is reduced in primary cortical neurons (Fig 3.28). This threshold effect is surprisingly high given previous results have shown a threshold as low as 36% before respiration and ATP synthesis are affected (Pathak and Davey, 2008) suggesting the process of mitochondrial fusion is robust and is only halted in cases of severe cellular stress. Interestingly, $\Delta\psi_m$ is also significantly reduced after a 5 min incubation of 20 nM piericidin A, yet ATP production is not affected until incubation with 100 nM piericidin A suggesting the inhibition of fusion is driven by $\Delta\psi_m$ -dependent cleavage of Opa1 as shown by the decrease in ratio of L-Opa1:S-Opa1 in Fig 3.29. In addition to this, we see mitochondrial fragmentation at concentrations above 17.5 nM piericidin and this is correlated with a co-localization of Drp1 with mitochondria as shown in Fig 3.30. These results suggest a similar threshold effect is evident for mitochondrial fusion, through cleavage of Opa1, and mitochondrial fission, through activation (phosphorylation at S637) of Drp1. This observation links mitochondrial fission/fusion dynamics with bioenergetics dysfunction in neurons and may link the slow onset of symptoms of neurodegenerative disease as neuronal cell death does not occur until this threshold is met.

Chapter 3 highlights the potential for a therapeutic which may be beneficial in treatment of disorders affecting complex I function, Ndi1. Transfection of Ndi1 into primary cortical neurons restored mitochondrial fusion to control levels when complex I was inhibited with 50 nM piericidin A, a concentration which completely abolishes fusion rates in control cells. This demonstrates the ability of yeast NADH dehydrogenase to compensate for a deficit of electron transfer when mammalian CI is inhibited by and maintain mitochondrial fusion. Further research on delivery of this construct to specific brain regions and safety are required but

this may provide some therapeutic benefit in patients with diseases with obvious CI defects, such as PD.

Chapter 3 also examined the effect of toxins (MPP⁺) and proteins (α -synuclein) associated with PD on mitochondrial dynamics in differentiated PC12 cells. MPP⁺ has been associated onset of parkinsonian like symptoms for many years (Hala et al., 1983) and has been shown to inhibit complex I, reduce mitochondrial transport and increase ROS production in cells (Cartelli et al., 2010, Piao et al., 2012). Our results demonstrate a 20 min pre-incubation with this toxin also reduces mitochondrial fusion in differentiated PC12 cells (Fig 3.32). This effect is not surprising considering the well documented inhibition of CI by MPP⁺ and the results presented in chapter 3. However, in contrast to the inhibition seen with etomoxir and UK5099, this effect appears to be mediated through L-Opa1 cleavage to S-Opa1 from preliminary results. This result suggests that deficits in mitochondrial fusion over time in neurons of the substantia nigra may be a causative factor in the onset of parkinsonian symptoms. Lending further weight to this hypothesis, overexpression of both wild type and mutant (A53T) α -synuclein in differentiated PC12 cells decreases mitochondrial fusion. Previous studies have shown that α -synuclein overexpression can induce mitochondrial network fragmentation (also seen here) as a result of Drp1 localization to the mitochondrion. Our results showing that mitochondrial fusion is also affected by overexpression of wild type and mutant α -synuclein highlight that the two processes are inextricably linked and slight perturbations over time may have cumulative effects in the dysfunction of mitochondrial transport and bioenergetics and may play a causative role in the onset of PD.

Chapter 4 of this thesis examines the effect of substrate transport into the mitochondrion on mitochondrial dynamics, especially the potential of a role for fatty acid oxidation in maintenance of mitochondrial fusion. It is generally accepted that neurons use glucose, and lactate derived from neighboring glial cells, exclusively as a substrate for ATP synthesis. Using a neuronal model, this chapter shows that inhibition of CPT1, the rate limiting enzyme in fatty acid

oxidation, can decrease mitochondrial fusion in a dose dependent manner (Fig 4.2, 4.3 and 4.4). In addition to this, blocking pyruvate transport into the mitochondrion reduces mitochondrial fusion (Fig 4.5) and inhibition of both CPT1 and the MPC has a cumulative effect, decreasing mitochondrial fusion by over 60% in differentiated PC12 cells (Fig 4.6). Preliminary results suggest there is no significant alterations in mitodynamin expression after treatment with these compounds, suggesting mitochondrial fusion can be directly affected by substrate availability. Further evidence for the utilization of fatty acids as a primary energy source in differentiated PC12 cells comes from the result that co-incubation with DCA, an inhibitor of PDK and thus activator of PDH, can overcome the deficits in fusion seen after treatment with 100 μ M etomoxir. Thus, when one energy source is compromised, a pharmacological increase in the transport of pyruvate into the mitochondrion can restore normal fusion rates. It would be interesting to examine the effects of co-treatment with DCA and etomoxir on respiration and $\Delta\psi_m$ to determine if these effects are specific to mitochondrial fusion or more general cellular bioenergetics. The finding that substrate transport can directly influence mitochondrial dynamics without affecting mitodynamin expression or post translational modification is important as recent research has suggested Opa1 and Drp1 are the link between bioenergetics and mitochondrial dynamics (Mishra and Chan, 2016), a concept which further weight is added to by the results in chapter 3. The results presented in chapter 4 open up debate as to whether other co-factors could alter the activity of the well-categorized mitodynamins resulting in subtle changes in mitochondrial fusion, without the “all or nothing” effect seen with Opa1 cleavage. Aside from the GTP-dependence of the mitodynamins, both kinesin and dynein are ATPases (Johnson and Wall, 1983, Kuznetsov and Gelfand, 1986), and so it is conceivable that energy deficiency as a result of reduced substrate availability prevented mitochondrial transport, reducing the potential for mitochondrial fusion events. It is imperative to examine the effect of these compounds in primary neurons to provide a clearer view of the potential for use of fatty acid oxidation as an energy source to drive mitochondrial fusion in neural cells.

O-GlcNAcylation of protein targets has emerged as a prominent area of neurodegenerative research in recent years as it has been shown that the enzymes responsible for O-GlcNAc cycling are highly expressed in the brain and their activities are age dependent (Wani et al., 2016). Chapter 5 examined the effect of pharmacological and siRNA mediated inhibition of these enzymes on mitochondrial dynamics and bioenergetic function in differentiated PC12 cells. It was found that pharmacological inhibition of OGA decreased mitochondrial fusion (Fig 5.4), likely as a result of decreased motility, as reported by Pekurnaz et al. (2014). However, this result was not replicated when OGA was stably knocked down (Fig 5.12) despite obvious changes in mitochondrial morphology (Fig 5.19). In contrast, pharmacological inhibition of OGT had no effect on fusion rates but knockdown resulted in a slight increase (Fig 5.13). Significant changes were seen in $\Delta\psi_m$ and ROS production in knockdown cells as OGA knockdown significantly increased $\Delta\psi_m$ while OGT knockdown significantly decreased $\Delta\psi_m$ (Fig 5.15) and increased ROS production (Fig 5.17). In agreement with this, respiration was significantly increased in OGA knockdown and decreased in OGT knockdown cells (Fig 5.20), while CI activity was increased in OGA knockdown cells (Fig 5.24).

These results demonstrate the myriad of cellular effects altering O-GlcNAcylation levels has in differentiated PC12 cells. Much of the data published on this subject so far is conflicting and highlights the varying and subtle effects this PTM has in different cell types. Research has focused on cardiac myocytes but in the brain some observations have pointed to a protective effect of increasing O-GlcNAcylation by decreasing the toxicity of protein oligomers while other observations have suggested that this may decrease protein clearance and induce apoptosis. The results from the literature point towards maintaining a balance of O-GlcNAc cycling as being key to maintaining neuronal homeostasis and subtle targeting of the cycling of O-GlcNAc may provide some therapeutic benefit in the future. The results presented in chapter five focus on the effect of O-GlcNAcylation on metabolic regulation within the cell and suggest that an increase in global O-GlcNAc levels increases the metabolic capabilities of the cell.

In theory, this agrees with the hypothesis that metabolic dysfunction is prevalent in older individuals with neurodegenerative diseases who display decreased levels of brain O-GlcNAcylation (Liu et al., 2012b, Deng et al., 2009). Chapter 5 provides a study on the overall effect of altering O-GlcNAc levels on mitochondrial function which broadly agrees with the hypothesis proposed by Banerjee et al. (2015). This proposal suggests Inhibition of OGT has effects which are more specific to mitochondrial metabolism where as OGA inhibition appear to have an effect on overall cellular metabolism. It will be interesting to investigate this further and confirm if there is a role for O-GlcNAc cycling in controlling the activity of a metabolic master regulator.

Chapter 6 demonstrated that the cell surface glycome of primary cortical neurons can be metabolically labelled using modified azido sugars and subsequently imaged using super resolution microscopy. Using STED microscopy we were able to visualize the incorporation of sialic acids in neurons and identify heavily sialylated ring and raft structures on the cell surface (Fig 6.12). It has previously been shown that ion channels are sialylated on the extracellular side (Hall et al., 2011, Cronin et al., 2005) and due to the ring structures identified we hypothesize that the STED images allow for visualization of ion channel complexes, although further work is needed to confirm this. In addition to this, this chapter examined how inhibition of a variety of different glycosyltransferases effects the incorporation of three modified azido sugars into the cell surface glycome. From the subsequent imaging, it was possible to determine that *N*-glycosylation is the predominant form of glycosylation in this type of neuron. We were also able to elucidate that GlcNAz (and subsequently endogenous GlcNAc) is present in both *O*- and *N*-glycans as well as in sphingolipids and glycoproteins clustered around the cell soma. GalNAz is present only in the *N*-glycans of glycoconjugates while ManNAz is also only present in *N*-glycans but appears to be found primarily on glycoproteins. These results provide scope for identifying some of the key glycans involved in neuronal differentiation and function as this PTM is thought to play important roles in neurotransmission and neural development.

7.2 Future Work

The work presented in chapter 3 demonstrates that inhibition of the ETC abolishes mitochondrial fusion in differentiated PC12 cells and primary neurons through a mechanism that cleaves Opa-1 as a result of a decrease in $\Delta\psi_m$ and/or decreases in cellular ATP levels. While this mechanism accounts for the inhibition of fusion seen, it is important to confirm that Drp1 is activated in response to ETC inhibition in order to explain the rapid fragmentation that occurs after treatment with inhibitors. This could be done by isolating mitochondria from PC12 cells and primary cortical neurons after treatment with the inhibitors and comparing levels of Drp1 in the cytosolic fraction and the mitochondrial fraction. One would expect a high degree of mitochondrial localization after treatment with inhibitors based on the results presented in chapter 3 and the imaging results which show a co-localization of Drp1 and NDUFA9. It would also be possible to look at Drp1 activation through western blotting using a phospho-Drp1 (S637) antibody which is commercially available. Examination of intracellular Ca^{2+} flux may provide further insight into the activation of Drp1 as OXPHOS inhibition has been shown to result in calcium efflux from mitochondria. Ca^{2+} flux can be measured by a number of commercial kits and increases after OXPHOS treatment would provide a mechanism of Drp1 activation as calcineurin is known to dephosphorylate Drp1 at S656, resulting in its activation.

This experiment would also be useful to carry out on primary neurons treated with the varying concentrations of piericidin A. It would be interesting to examine if the fusion threshold is aligned with a threshold for maintenance of supercomplexes in primary neurons. A balance of L-Opa1 and S-Opa1 has been shown to be important in maintaining cristae tightness and supercomplex assembly. It is conceivable that treatment with 20 nM piericidin A would cleave L-Opa1 and promote supercomplex disassembly, further linking healthy mitochondrial dynamics with bioenergetic function. This could be examined through blue native polyacrylamide gel electrophoresis (BN-PAGE) on mitochondrial fractions obtained from treated neurons. Finally, it would be pertinent to confirm that

transfection with Ndi1 is able to prevent the cleavage of Opa1 and the decrease in $\Delta\psi_m$ observed after treatment with concentrations of piericidin A above 20 nM. Unfortunately, due to low levels of expression and high toxicity associated with transfection of primary neurons it was not possible to carry out these experiments. However, it may be possible to express Ndi1 using a lentiviral method which would reduce toxicity and allow enough cells to be harvested for western blot/imaged with TMRM. This would definitively confirm that Ndi1 rescues fusion in neurons where CI is inhibited by greater than 53% by a mechanism that prevents Opa1 cleavage.

Chapter 4 demonstrated that inhibition of substrate transport into the mitochondrion can decrease mitochondrial fusion. Preliminary experiments suggest that this is not as a result of Opa1 cleavage but this must be repeated a number of times to confirm this is the case. It would be useful to assess cell viability after longer treatments with the inhibitors used in chapter 4 to ensure there is no toxicity, particularly at higher concentration of etomoxir. If it is confirmed that mitochondrial fusion is decreased through an Opa1 independent mechanism, this area will be open to much greater investigation. It will be useful to examine $\Delta\psi_m$ in cells after treatment, although one would not expect significant changes in this if Opa1 is not cleaved. More important may be the measurement of rates of oxygen consumption and extracellular acidification to determine if a change in the rate of respiration is responsible for the decrease in fusion in differentiated PC12 cells. Previous work in the Davey lab has shown that α -synuclein can inhibit CI in synaptic and non-synaptic mitochondria (unpublished data). It would be useful to carry out CI activity assays on homogenates from the wild type and mutant (A53T) α -synuclein overexpression cells to determine if this is the case in a cellular model. This may help to clarify the threshold effect of CI inhibition on mitochondrial fusion, although it is likely the threshold is different in an immortalized cell line than primary cortical neurons.

Chapter 5 provided an insight into the effect of altering global cellular levels of O-GlcNAcylation on mitochondrial function in a neuronal model. The results

presented here were in conjunction with another student who has examined the effect of altering O-GlcNAc levels in synaptosomes and non-synaptic mitochondria giving a broad picture of the influence this PTM has on mitochondrial function in brain. Despite the depth of this study there is still much to understand about the effect of O-GlcNAcylation. It would be useful to measure ATP content of the knockdown cells to determine if the differences seen in respiration and complex activities result in increased/decreased ATP production. As mentioned in section 5.4, a measurement of the RCR using mitochondria isolated from the knockdown cells or synaptosomes treated with thiamet G and TT04 should be carried out to determine the respiration state of mitochondria in which O-GlcNAc levels are altered. Perhaps most importantly, it would be interesting to isolate mitochondria and carry out an immune pull down assay followed by proteomic analysis to determine the mitochondrial proteins which are O-GlcNAcylated. This would likely yield more insight into the changes in bioenergetics function and mitochondrial dynamics we are seeing after pharmacological and siRNA mediated inhibition of the enzymes responsible for O-GlcNAc cycling.

This thesis has provided some insight into possible mechanisms that contribute to mitochondrial dysfunction in the brain and highlights the extreme vulnerability of neurons to slight perturbations in mitochondrial dynamics and bioenergetics. It provides a basis for further investigation into the role of mitochondrial dynamics in progression of neurodegenerative disease and the role of O-GlcNAc cycling in mitochondrial function in a neuronal model.

7.3 Conclusions

This thesis aimed to investigate the mechanisms that may contribute to the dysfunctions in mitochondrial dynamics seen in many neurodegenerative diseases by studying the effects of ETC inhibition, substrate transport and altered O-GlcNAc cycling on mitochondrial function. The findings presented here are the following:

- Inhibition of the ETC in differentiated PC12 cells abolishes mitochondrial fusion via a mechanism dependent on Opa1 cleavage
- Inhibition of complex I by greater than 53% in primary cortical neurons results in a complete cessation of mitochondrial fusion via a mechanism dependent on Opa1 cleavage which can be rescued by Ndi1
- Maintenance of substrate transport into the mitochondria is vital for maintaining normal mitochondrial fusion
- Knockdown of the enzymes responsible for O-GlcNAc cycling has major effects on mitochondrial function, likely via a variety of targets
- The cell surface glycome of primary cortical neurons can be metabolically labelled using modified azido sugars and this glycome is composed of predominantly *N*-glycans

8. Bibliography

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