Disruption of Kif5c Mediated Movement Affects Mitochondrial Form, Function and Cellular Health in Rat Primary Cortical Neurons and Astrocytes

by
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B.Sc.(Hons), Simon Fraser University, 2011

Thesis Submitted In Partial Fulfillment of the Requirements for the Degree of Master of Science

in the Department of Biological Sciences Faculty of Science

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Abstract

A clear understanding of mitochondrial dynamics is important in neurodegenerative diseases. Due to the unique architecture of neurons, trafficking of mitochondria throughout processes to regions of high energetic demand is critical to sustain neuronal health. I evaluated the consequences of disrupted kif5c mediated mitochondrial movement on form and function. Morphology and cellular distribution of mitochondria were affected predominantly due to remodelling, which resulted in punctate shaped mitochondria. We also demonstrated that punctate mitochondria resulted in decreased ROS and increased ATP cellular levels. Neurons overexpressing the dominant negative form of kif5c exhibited greater survival than controls following excitotoxicity, suggesting that the kinesin mutant conferred some form of neuroprotection. Our results suggest a novel role of kif5c. In addition to mediating mitochondrial transport, kif5c plays a role in the mechanism of regulating mitochondrial morphology. Our results suggest that kif5c mediated mitochondrial dynamics may play an important role in regulating various mitochondrial functions and in turn cellular health.

Keywords: Kif5c; Mitochondrial movement; mitochondrial dynamics; mitochondrial remodelling; ATP; ROS
Dedication

I would like to dedicate this to my Mom and Dad, Georgina and Promise Iworima, for always being supportive of my endeavours in science and for believing in me every step of the way. I know you are watching after me from heaven mommy. I hope I have made you proud. I love you.
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<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>APOER2</td>
<td>Apolipoprotein E receptor 2</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>ATeam</td>
<td>Adenosine Triphosphate indicator based on ε subunit for analytic measurement</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan fluorescent protein</td>
</tr>
<tr>
<td>CMT2A</td>
<td>Charcot Marie Tooth 2 A</td>
</tr>
<tr>
<td>DCFDA</td>
<td>5-carboxy-2’,7’-dichlorofluorescein diacetate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRP1</td>
<td>Dynamin-related protein 1</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>FCCP</td>
<td>Trifluorocarbonylcyanide Phenylhydrazone</td>
</tr>
<tr>
<td>FIS1</td>
<td>Fission protein 1</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>GRIF1</td>
<td>γ-aminobutyric acid A receptor–interacting factor-1</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HBSS</td>
<td>HEPES buffered saline solution</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>KCN</td>
<td>Potassium cyanide</td>
</tr>
<tr>
<td>KHC</td>
<td>Kinesin heavy chain</td>
</tr>
<tr>
<td>KLC</td>
<td>Kinesin light chain</td>
</tr>
<tr>
<td>MCU</td>
<td>Mitochondrial calcium uniporter</td>
</tr>
<tr>
<td>MFN1</td>
<td>Mitofusin 1</td>
</tr>
<tr>
<td>MFN2</td>
<td>Mitofusin 2</td>
</tr>
<tr>
<td>NB+AO</td>
<td>Neurobasal plus antibiotics</td>
</tr>
<tr>
<td>NB-AO</td>
<td>Neurobasal minus antibiotics</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>OIP106</td>
<td>O-linked N-acetylglucosamine–interacting protein 106</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>OPA1</td>
<td>Optic atrophy 1</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDL</td>
<td>Poly-D-lysine</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced kinase 1</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ro-GFP</td>
<td>Redox sensitive GFP</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>TPR</td>
<td>Tetratricopeptide repeat</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin proteasome system</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule organizing centre</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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1. Introduction

Neurons are highly specialized, post-mitotic cells involved in neurotransmission. They are polarized cells with a unique architecture: a cell body containing the nucleus, and elaborated processes (i.e. dendrites and axons) that extend from the cell body. Neurons typically have multiple dendrites. Incoming nerve signals are received at the postsynaptic membrane of dendrites. These signals pass through the soma for integration and then generation of an action potential. The action potential propagates down the axon towards the presynaptic terminal resulting in the release of neurotransmitters into the synaptic cleft. Neurons are highly excitable cells that require high levels of energy to meet metabolic needs. The intrinsic properties of mitochondria make them crucial for proper neuronal function. Mitochondria are double membraled organelles that contain an outer membrane, intermembrane space and an inner membrane. The inner membrane borders the mitochondrial matrix and forms invaginations referred to as cristae, resulting in increased surface area. The human mitochondrial genome is a circular molecule of 16,596 base pairs which encodes for 13 protein components of the electron transport chain, 22 tRNAs and 2 rRNAs (Anderson et al., 1981). The remainder of the mitochondrial proteins are encoded by the nuclear genome and are imported into the mitochondria from their point of origin (Asin-Cayuela and Gustafsson, 2007).

Mitochondria are referred to as the power house of the cell. They are essential for production of Adenosine Triphosphate (ATP) via oxidative phosphorylation, for the maintenance of calcium homeostasis by buffering intracellular calcium, and are major generators of reactive oxygen species (ROS). Following mitochondrial biogenesis in the cell body, mitochondria are trafficked to regions of high metabolic demand, such as active growth cones, synaptic terminals and nodes of Ranvier (Davis and Clayton, 1996; Morris and Hollenbeck, 1993; Rintoul and Reynolds, 2010). As neuronal development progresses, neuronal processes elongate. Neurons face the challenge of transporting and delivering mitochondria across large distances, and must manage the large,
dynamically changing demand for ATP and local Ca\(^{2+}\) regulation at the various synaptic sites in neuronal processes (Chang et al., 2006a).

Mitochondria are dynamic, semi-autonomous organelles. They are able to fuse together to form a single mitochondrion. Mitochondrial fusion is facilitated by the proteins mitofusin 1/2 (mfn1, mfn2) and optic atrophy 1 (OPA1) (Chen et al., 2003; Delettre et al., 2000; Rintoul and Reynolds, 2010). Mitochondria also undergo fission where a single mitochondrion is divided into two or more daughter mitochondria, a process mediated by the GTPase dynamin related protein (drp1) and fission protein 1 (fis1) (Lee et al., 2007; Yu et al., 2005). Mitochondria appear as threadlike elongated organelles; this morphology is maintained by the balance between the fusion and fission machineries (Rube and van der Bliek, 2004). Because the electron transport chain of mitochondria is the major source of intracellular ROS production, mtDNA are particularly vulnerable to ROS-induced mutations as a result of the close proximity to the source of ROS generation. An accumulation of mutated mtDNA leads to the dysfunction of the functional respiratory chain complexes which perturbs cellular health and ultimately causes cell death (Westermann, 2010). Mitochondria are also recycled and degraded by a unique autophagy pathway called mitophagy (Lemasters, 2005). Mitophagy serves as a quality control for damaged mitochondria, as well as maintaining a steady-state via regulation of mitochondrial turnover.

The processes of mitochondrial fission, fusion, recycling and movement can be collectively termed mitochondrial dynamics. The maintenance of the proper functions of these mitochondrial dynamics is important for cellular health and dysfunction has been implicated in numerous pathophysioologies associated with diseases.

1.1. Mitochondrial Morphology

1.1.1. Fusion

Mitochondrial fusion is the process where at least 2 individual mitochondria fuse together to form a single mitochondrion. Mitochondrial fusion is mediated by the activities of mitofusins 1 and 2 (mfn1 and mfn2) in conjunction with optic atrophy protein 1 (OPA1) (Chen et al., 2005; Chen et al., 2003; Cipolat et al., 2004). Mfn1 and mfn2 are
large GTPase transmembrane proteins localized to the outer mitochondrial membrane. They are required for the tethering of the outer mitochondrial membrane during fusion (Koshiba et al., 2004). OPA1 is also a GTPase located in the mitochondrial intermembrane space essential for fusion of the inner mitochondrial membrane (Chen and Chan, 2005; Cipolat et al., 2004; Griparic et al., 2004). In mouse embryonic fibroblasts expressing null mfn1 or mfn2 genes, mitochondria were completely fragmented and exhibited no detectable signs of fusion (Koshiba et al., 2004). Conversely, when OPA1 was overexpressed in COS-7 cells, mitochondrial morphology became more elongated as a result of upregulation of the fusion machinery (Cipolat et al., 2004). Mutations to OPA1 are associated with autosomal dominant optic atrophy (ADOA) which is characterized by a progressive loss of visual acuity (Alexander et al., 2000a; Alexander et al., 2000b). Likewise, mutations to mfn2 have been implicated in the pathology of Charcot-Marie tooth 2A (Baloh, 2008; Baloh et al., 2007). Application of neurotoxin has also resulted in a change to mitochondrial morphology (see discussion for details) (Malaiyandi et al., 2005; Rintoul et al., 2006; Rintoul et al., 2003). The links between dysfunctional mitochondrial morphology proteins and subsequent mitochondrial dysfunction demonstrates the importance of mitochondrial morphology regulation to neuronal function and overall cellular health.

Juxtaposition of mitochondria is necessary to facilitate mitochondrial fusion; therefore a functional trafficking network is essential. Because fusion necessitates the mixture of intramitochondrial content, fusion may serve to restore normal function to damaged mitochondria by mixing with healthy mitochondria (Chen and Chan, 2005). As a result, damaged mtDNA repairs can be promoted through the redistribution of mitochondrial proteins between damaged and healthy cells thereby preventing the initiation of apoptotic cascades and cellular dysfunction (Chen et al., 2005). Mitochondria in motion have a higher rate of fusion compared to immobile mitochondria (Liu et al., 2009). Impairment of mitochondrial trafficking may impede the occurrence of fusion events leading to the accumulation of dysfunctional mitochondrial. Accumulation of dysfunctional mitochondria is a characteristic of numerous diseased states (Kowald, 1999; Osellame and Duchen, 2013).
1.1.2. Fission

Mitochondrial fission or fragmentation is the process where a single mitochondrion divides into 2 or more smaller daughter mitochondria. The key molecules involved in mitochondrial fission are the dynamin-related protein (drp1) and fission protein 1 (fis1) (James et al., 2003; Smirnova et al., 2001). Drp1 is a protein largely distributed in the cytosol that is recruited to the outer mitochondrial membrane to promote fission that hydrolyzes GTP to mediate mitochondrial fission (Varadi et al., 2004). Drp1 is phosphorylated at Ser656 by the cyclic AMP-dependent protein kinase; the phosphorylation of drp1 prevents mitochondrial fission (Cribbs and Strack, 2007). Conversely, drp1 is dephosphorylated by the phosphatase calcineurin, resulting in the relocation of drp1 from the cytoplasm to mitochondria (Cribbs and Strack, 2007). Drp-1 is thought to polymerize and form a ring-like structure that constricts the mitochondrion and eventually split it into 2 daughter organelles (Hoppins et al., 2007). Fis1 is a protein containing a tetratricopeptide repeat (TPR) domain for protein-protein interaction and is anchored to the outer mitochondrial membrane (Koch et al., 2005). Overexpression of fis1 resulted in fragmentation of the mitochondrial network in COS-7 cells, supporting its role in fission (James et al., 2003). Although the mechanism regulating the recruitment of cytoplasmic drp1 remains unclear, fis1 is suspected to be involved in translocation of drp1 from the cytoplasm to the mitochondrial membrane (Lee et al., 2007; Shaw and Nunnari, 2002; Yoon et al., 2003; Yu et al., 2005).

Fission may play a role in segregation of debris and misfolded proteins in damaged mitochondria before degradation (Sheng and Cai, 2012; Youle and van der Bliek, 2012). In young primary cortical neurons, mitochondria were found to be shorter and more motile compared to mature neurons with longer less motile mitochondria (Chang and Reynolds, 2006a). The increased motility observed in immature neurons is proposed to be important to support active protein synthesis, axon elongation and synaptogenesis crucial for normal health and physiology of neurons at that stage in development.
1.1.3. Remodelling

Remodelling is rapidly becoming appreciated as a distinct mitochondrial dynamic whose mechanism remains unclear. Remodelling or rounding up of mitochondria exhibits similar physical characteristics to those observed during fragmentation; mitochondria are shortened and punctate. However, remodelling does not result in the division of a single mitochondrion into daughter mitochondria.

Fragmentation/fission and remodelling are mediated by an increase in intracellular calcium (Tan et al., 2011). Activation of the phosphatase calcineurin by calcium results in the dephosphorylation of drp1; this facilitates the translocation of cytoplasmic drp1 to mitochondria and subsequent fission (Cereghetti et al., 2008; Chang and Blackstone, 2007; Cribbs and Strack, 2007). Elevation of intracellular calcium in cortical astrocytes by the Ca$^{2+}$ ionophore 4Br-A23187 resulted in increased mitochondrial count and decreased length, consistent with fission events (Tan et al., 2011). When calcineurin inhibitors (cyclosporin A (CsA), or tacrolimus (FK506)) were added in parallel with 4Br-A23187, it appeared that only fission was blocked, and not remodelling. The effects of 4Br-A23187 on mitochondrial length remained largely unaltered; mitochondrial length was still reduced, however, there was a significant decrease in the mitochondrial count in the presence of the calcineurin inhibition compared to cells without the inhibitor. These results are consistent with mitochondrial remodelling and suggest a distinct pathway besides fission, that results in the shortening of mitochondria. Similar remodelling has been reported in neurons (Han et al., 2008b; Rintoul et al., 2003), but this phenomena remains widely uncharacterized and many authors fail to distinguished this phenomenon from fission.

1.2. Mitochondrial Movement

Intracellular movement of mitochondria in neurons is important for cellular health and function. Although the functional significance of mitochondrial motility has not been fully elucidated, movement has been implicated in mitochondrial distribution and morphology (Boldogh and Pon, 2007). Mitochondria move in different directions at different speeds, they oscillate and also exhibit saltatory movement and can
spontaneously pause and change their trajectory (Chang and Reynolds, 2006b; Rintoul et al., 2003). Movement of mitochondria is mediated by the interaction between 3 major groups of proteins: cytoskeletal elements, molecular motor proteins and adaptor proteins that aid cargo loading and docking (Chevalier-Larsen and Holzbaur, 2006).

Mitochondria are transported on microtubules, which mediate long distance trafficking, and actin microfilaments, for travel over shorter distances (Eschbach and Dupuis, 2011; Huang et al., 1999). Microtubule elements consist of α/β tubulin subunits. The minus end is anchored on the microtubule organizing centre (MTOC) in the soma and extends outwards by polymerization on the plus end (Luders and Stearns, 2007). Further evidence of the distinct architecture of neurons is exhibited in the polarity of microtubules in axons versus dendrites. Orientation of microtubule tracks within the axon has the plus end directed towards the periphery, away from the soma. However, in the dendrites, microtubules are in a mixed orientation. In mature neurons microtubules are less dynamic as a result of stabilizing microtubule associated proteins (MAPs). Microtubule-dependent mitochondrial movement is bidirectional and mediated by two ATPase molecular motors: kinesins and dynein (Tanaka et al., 1998). The axon lacks protein synthesis machinery; therefore, proteins must be transported from the site of synthesis, usually the soma, down to the periphery, as well as to synaptic terminals (Hirokawa, 1998). Various neurotoxins have been shown to cause cessation of mitochondrial movement, and sometimes altered mitochondrial shape (Chang et al., 2006b; Malaiyandi et al., 2005; Rintoul et al., 2003). Aggregation of the huntingtin ( htt) protein in neuronal processes blocks the transport of other proteins and organelles (including mitochondria) by acting as a physical road block. This blockade results in further accumulation of mitochondria potentially leading to bioenergetic failure associated with Huntington’s disease and gradual neurodegeneration (Li et al., 2001; Li et al., 2003). Neurons with aggregated htt also exhibit increased susceptibility to excitotoxicity (Chang et al., 2006b). The importance of mitochondrial transport is emerging as an essential aspect involved in the pathology of other neurodegenerative diseases thus demonstrating its role in the maintenance of cellular function (see section 1.4)(Balo et al., 2007; De Vos et al., 2008; Sheng and Cai, 2012)
1.2.1. **Kinesin**

Kinesins belong to a superfamily of proteins that mediate anterograde (microtubule plus end directed) movement of cargo. The kinesin-1 subfamilies (kif5a, kif5b, kif5c) are responsible for the movement of mitochondria in neurons (Hirokawa, 1998). Kif5a and kif5c are neuron specific whereas kif5bc is expressed ubiquitously in many other tissues (Kanai et al., 2000; Miki et al., 2001).

Kinesin-1 is a heterodimer that consists of two heavy chains (KHC) and two light chains (KLC) (fig. 1a) (Howard, 1996). Each KHC also contains a highly conserved N-terminal motor domain, also referred to as the head region, which has 2 parts: the globular catalytic core and a microtubule binding motif (Vale and Fletterick, 1997). The catalytic core, which could be located at the N- or C- terminus of the neck region, contains an ATP-binding sequence that is required for the binding and hydrolysis of ATP necessary for movement (Chevalier-Larsen and Holzbaur, 2006). The microtubule binding motif of the KHC interacts with the tubulin subunits of microtubules and undergoes an 8nm stepwise displacement via a hand-over-hand mechanism for each ATP hydrolysis cycle (Carter and Cross, 2005; Endow et al., 2010; Svoboda et al., 1993). Kinesin-1 is highly processive; once kinesin-1 is bound to microtubules, it travels long distances spanning over 100 tubulin subunits before detaching and thereby enabling long-range transportation of cargo (Rice et al., 1999; Vale et al., 1996).

Kinesin-1 also has a stalk domain in between the KHC and KLC. It contains an α-helical coiled-coil that forms a flexible hinge and mediates dimerization of the KHC (Vale and Fletterick, 1997). The C-terminal KLC has a cargo-binding domain, also known as the tail region, that is variable. This increases the possible number of cargoes transported by kinesins. Association of the cargo to the tail domain may be direct or indirect via scaffolding/adaptor proteins. The KLC contains TPR domains where cargoes or adaptor proteins bind (Coy et al., 1999). Interestingly, certain cargoes are capable of binding directly to the KHC, and it has been hypothesized that a cargo preferentially binding to the KHC results in translocation to the somatodendritic regions versus binding to the KLC which sorts to the axon (Hirokawa and Takemura, 2004; Setou et al., 2002). Kinesin mediated trafficking of mitochondria is coordinated by the binding of
mitochondria to the tail region of the motor, however, recruitment of mitochondria is facilitated by some adaptor proteins.

**Figure 1.** Schematic diagram of conventional kinesin-1 (kif-5)

(a) shows the different domain on the kif5 molecular motor which includes (b) regulation of kinesin mediated mitochondrial movement by calcium. Images were used with permission from their respective authors.

1.2.2. **Adaptor proteins: Milton and Miro**

Kinesin-1 trafficking of mitochondria in neurons is mediated by a motor/adaptor protein complex: the milton/miro/KHC complex (Rice and Gelfand, 2006). Milton was shown to co-immunoprecipitate with KHC and mitochondria in axons suggesting a role in regulating anterograde transport of mitochondria (Brickley and Stephenson, 2011;
Stowers et al., 2002). Milton is specifically involved in the recruitment of the KHC to mitochondria and competes with the KLC to bind mitochondria (Schwarz, 2013). Milton scaffolds KHC to mitochondria via the integral mitochondrial rho-like GTPase, miro (Glater et al., 2006). Miro has 2 GTP-binding domains with two EF hands capable of binding calcium. It also has a transmembrane domain that aids anchoring of miro to the outer mitochondrial membrane while the functional domains remain exposed (Fransson et al., 2006). Two mammalian isoform of milton have been identified: milton1 and 2, also known as O-linked N-acetylglucosamine–interacting protein 106 (OIP106) and γ-aminobutyric acid A receptor–interacting factor-1 (GRIF-1) (Brickley et al., 2005). Likewise, there are also 2 mammalian forms of miro: miro1 and 2 (Fransson et al., 2006).

Because neurons are highly active cells, the regulation of mitochondrial distribution to various regions of high metabolic demand is important for cellular health. Expression of miro in drosophila neuronal cultures revealed that milton co-localized with miro and was essential for the transport and proper subcellular distribution of mitochondria in dendrites and axon terminals (Guo et al., 2005). Intracellular calcium has been demonstrated to regulate mitochondrial movement (Chang et al., 2006a; Rintoul et al., 2003; Yi et al., 2004). This calcium dependent mechanism of mitochondrial movement is mediated by the calcium binding EF hands of miro (fig. 1b) (Wang and Schwarz, 2009). When the EF hand were mutated in miro, elevated levels of calcium failed to inhibit axonal mitochondrial trafficking observed in control cells expressing wild-type miro. During the calcium mediated arrest of mitochondrial movement, calcium binds to the EF hands of miro; this results in an interaction between miro and the KHC, thus preventing the interaction of kinesin with microtubules (Wang and Schwarz, 2009). The KHC dissociates from microtubules. In addition to its effects on mitochondrial trafficking, elevated intracellular calcium has also been shown to increase mitochondrial fragmentation and over-stimulation of calcium dependent degradation pathways (Rintoul et al., 2003; Trapp and Stys, 2009).

1.2.3. Dynein

Axonal retrograde trafficking of mitochondria towards the minus end of microtubules is mediated by the dynein superfamily protein. Cytoplasmic dynein is a
multi-subunit complex composed of heavy chains that constitute the motor domain and an ATP-binding domain, intermediate chains composed of non-catalytic subunits, and light chains (Gee et al., 1997; Hirokawa, 1998). Dynein also has an important associated protein called dynactin, which contains p150\textsuperscript{glued}, a coiled-coiled protein that binds directly to dynein intermediate chains and microtubules (Chevalier-Larsen and Holzbaur, 2006; Eschbach and Dupuis, 2011; Hirokawa et al., 2010; Waterman-Storer et al., 1997). Dynactin also binds to mitochondria and increases dynein processivity (King and Schroer, 2000; Schroer, 2004). Similar to kinesin-1 once bound, the dynein/dynactin complex remains attached for a considerable distance before detachment. The step size of dynein is highly variable between 4-32nm, but is predominantly 8nm (Gennerich and Vale, 2009; Toba et al., 2006; Yildiz et al., 2004).

Dynein/dynactin complex dysfunction leads to accumulation of damaged mitochondria at the cell periphery; it also causes late-onset motor neurodegeneration in transgenic mice similar to human amyotrophic lateral sclerosis (ALS) (LaMonte et al., 2002). Apposition of individual mitochondria is a prerequisite for a fusion event (see section 1.1.1.). Perturbation of the p50 subunit of the dynein/dynactin complex results in the redistribution of mitochondria and a change in their morphology to a more elongated form in HeLa cells, which may be due to increase in fusion or reduction in fission events (Varadi et al., 2004). Interestingly, there was also translocation of drp1 from mitochondria to the cytosol. Because drp1 is a non-resident mitochondrial membrane protein, these results suggest a possible novel role of dynein/dynactin in targeting cytoplasmic drp1 to mitochondria, and consequently influencing mitochondrial morphology by governing fusion and fission rates.

1.3. Mitophagy

Mitophagy is the selective removal of damaged mitochondria involving engulfment by a double membraned structure called the autophagosome and subsequent fusion to perinuclear-clustered lysosomes for catalytic degradation (Lemasters, 2005; Tolkovsky, 2009). Mitophagy is essential for quality control of mitochondria under physiological conditions, facilitating organelle turnover and protein degradation and recycling. In mammalian cells, the formation of a mature
autophagosome is preceded by the formation and/or elongation of an isolation membrane called the phagophore (Longatti and Tooze, 2009). This membrane expands around, and eventually encloses, the damaged mitochondrion and the resulting autophagosome is then trafficked to late endosomes or lysosomes where subsequent fusion of these organelles results in the formation of an autolysosome to complete the process (Kimura et al., 2007; Yamamoto et al., 2010). Although there are a set of autophagy-related (Atg) proteins involved in autophagosome formation, only a subset of them remain throughout maturation and can be used as markers (Kimura et al., 2008; Suzuki et al., 2007). One such protein Atg8, or microtubule-associated protein light chain 3 (LC3) in mammals, is a ubiquitin-like protein widely used to monitor mitophagy in cells (Nakatogawa et al., 2007).

Trafficking and fusion of autophagosomes to lysosomes require the microtubule network (Kochl et al., 2006). Experimental data have shown that cytoskeletal microtubules facilitate fusion between the autophagosome, which contains sequestered mitochondria, and lysosomes (Fass et al., 2006; Kochl et al., 2006; Webb et al., 2004). Depolymerization of microtubules with nocodazole abolished normal motility of autophagosomes following the induction of mitophagy by depletion of amino acids in hepatocytes. This resulted in decreased translocation of autophagosomes to lysosomal compartments and an associated reduction in protein degradation in the cells. Microtubules are also involved in the process of autophagosome formation (Fass et al., 2006; Kochl et al., 2006; Longatti and Tooze, 2009; Tooze et al., 2010). The number of starvation-induced autophagosomes was reduced following treatment with nocodazole; the rate of formation was also decreased, suggesting some dependence on microtubules.

In neurons, biogenesis of autophagosomes is believed to occur at the distal end of the axon (Hollenbeck, 1993). These autophagosomes are then retrieved via retrograde molecular motors and transported to the cell body where lysosomes are concentrated (Hollenbeck, 1993; Wang et al., 2006). Transportation of autophagosomes is dependent on dynein (Kimura et al., 2008). Following the stimulation of autophagosome formation in HeLa cells by amino acid starvation, trafficking towards lysosomes was detected. When the function of the dynein/dynactin complex was impaired by overexpression of dynamitin (p50), a subunit of dynactin, or microinjection of
anti-dynein intermediate chain antibody, rapid movement of autophagosomes to lysosomes close to the centre of the cell was significantly reduced (Kimura et al., 2008). It still remains unclear how the autophagosome is recruited to the dynein/dynactin complex for retrograde transport to commence.

The origin of the autophagosome remains controversial. One prominent hypothesis involves the autophagosome originating from a pre-existing organelle, the endoplasmic reticulum (Axe et al., 2008; Hamasaki and Yoshimori, 2010). Interestingly, dynein disruption not only prevents autophagosome-lysosome fusion, but is also involved in the early stages of autophagosome formation in U373 human glioma cells (Yamamoto et al., 2010). The supply of membrane for phagophore formation and maturation into the autophagosome may require microtubule based ER-to-Golgi transport (Yamamoto et al., 2010).

Although the complete mechanism of mitophagy remains unknown, various events have been identified to induce mitophagy. Experiments on hepatocytes revealed that photodamage as well as nutrient deprivation can serve as mitophagy initiators, confirmed by the co-localization of GFP-LC3 positive structures in areas of mitochondrial damage (Kim et al., 2007). A common feature of mitophagy is loss of the mitochondrial membrane potential. However, some studies support the notion that mitochondrial depolarization is necessary, but not sufficient for the induction of mitophagy (Kim et al., 2007; Rodriguez-Enriquez et al., 2006). Another potential trigger for mitophagy is the abnormal accumulation of ROS, a normal by-product of oxidative phosphorylation. Mitochondrial dysfunction can lead to increased ROS production, which in turn may trigger mitophagy (Scherz-Shouval et al., 2007). ROS appears to oxidize a cysteine protease, Atg4, directly at a cysteine residue close to the catalytic site, promoting the formation of the autophagosome and promote mitophagy (Scherz-Shouval et al., 2007).
1.4. Mitochondrial Dynamics (Fusion, Fission, Movement and Mitophagy) and Neurodegenerative Diseases

1.4.1. Parkinson’s Disease

Parkinson’s disease (PD) is the second most common neurodegenerative disease and is associated with the degeneration of dopaminergic neurons within the substantia nigra pars compacta. Its symptoms include bradykinesia, resting tremor, rigidity and an unsteady gait. It is widely held that malfunction of mitochondria contributes to the etiology of the disease (Valente et al., 2004). However, it is unclear whether disruption of mitochondrial dynamics (fission/fusion, movement, recycling/mitophagy) is the cause or result of the pathophysiology of PD.

Mutations in two genes, PINK1 and parkin, have been identified in recessive autosomal forms of Parkinson’s disease; however, the majority of PD cases are sporadic with unclear etiology (Deng et al., 2008b). Parkin is a cytosolic E3 ligase expressed ubiquitously throughout the cell, capable of tagging its substrate for degradation by the ubiquitin proteasome system (UPS) (Clark et al., 2006; Greene et al., 2003). PINK1, PTEN-induced kinase 1, is a Ser/Thr kinase that contains a mitochondrial targeting sequence at its N-terminus (Valente et al., 2004; Weihofen et al., 2009). PINK1 is imported into the mitochondrion where it undergoes a rapid membrane potential dependent proteolytic cleavage by presenilin-associated rhomboid-like protein (PARL) thereby maintaining low levels of PINK1 in healthy mitochondria (Jin et al., 2010). However, following a loss of membrane potential, PINK1 is stabilized on the outer mitochondrial membrane, and it recruits parkin towards the mitochondria (Matsuda et al., 2010). The recruitment of parkin leads to ubiquitination of the damaged mitochondria and triggers the mitophagy pathway.

Evidence suggests that PINK1 and parkin are involved in the machinery that regulates mitochondrial dynamics in Drosophila (Clark et al., 2006; Yang et al., 2008). PINK1 deficient transgenic male fruitflies exhibited apoptotic flight muscle degeneration, mitochondrial swelling and cristae fragmentation; however, there was no significant degeneration of dopaminergic neurons, one of the hallmarks of PD (Clark et al., 2006). Also associated with the drosophila PINK1 mutants was a decrease in ATP levels and
increased susceptibility to various stressors including oxidative stress. Decreased resistance to oxidative stress may be a result of mitochondrial malfunction as a result of increased ROS generation by the electron transport chain or a decrease in endogenous antioxidant levels. Similar results have also been observed in parkin deficient mutant fruitflies (Greene et al., 2003).

PINK1 and parkin also play a role in the fission/fusion machinery of mitochondria. As stated previously, experimental data suggest that mitochondrial morphology results from the balance between the activities of both fusion and fission proteins. The maintenance of this balance is important for the stability of mitochondrial bioenergetics and the distribution of mitochondria involved in dendritic spine maintenance, synaptic plasticity and synapse formation (Li et al., 2004; Twig et al., 2010). Overexpression of PINK1 in *drosophila* resulted in increased mitochondrial fission, and abnormal mitochondrial morphology in dopaminergic neurons, similar to the phenotype seen when drp1 was overexpressed (Yang et al., 2008). Although it is not fully understood how PINK1 mediates changes to mitochondrial morphology, it is speculated that Fis1 may mediate interactions between Drp1 and PINK1 leading to fission.

Parkin is also implicated in the fusion of mitochondria; overexpression of parkin was sufficient to rescue fragmented mitochondrial morphology in HeLa cells where parkin expression was silenced (Exner et al., 2007). Indeed, mitochondrial fission precedes and is essential for mitophagy (Twig et al., 2008). Fission events often generated two uneven mitochondrial daughters where one had a higher membrane potential that proceeded to subsequent fusion events and recovery of the membrane potential. The other daughter mitochondrion had lower membrane potential and OPA1 levels, thus reducing the probability of a fusion event and allowing for the more probable clearance by mitophagy. Remarkably, inhibition of the fission machinery or overexpression of OPA1 decreased mitophagy and resulted in reduced respiratory capacity and accumulation of oxidized mitochondrial proteins; this was independent of any increase in ROS production. Also, the prerequisite fission may serve to divide mitochondria into portions of manageable size for engulfment by the autophagosome (Youle and Narendra, 2011). This proposes another link between mitophagy and mitochondria fusion/fission.
1.4.2. Charcot-Marie Tooth Disease 2A

As mentioned previously, mitochondrial fusion is mediated by mfn1/2 proteins in conjunction with OPA1. Loss of mitochondrial fusion can lead to defective membrane potential, reduced oxidative phosphorylation and increased predisposition to cellular death (Chen and Chan, 2005). Dominant inheritance of mutations to mfn2 has been identified as causative for Charcot-Marie-Tooth disease subtype 2A (CMT2A). CMT2A is a peripheral neuropathy characterized by degeneration of long peripheral axons of motor and sensory neurons (Zuchner et al., 2004). Symptoms typically include progressive muscle weakness, sensory loss and eventually muscle atrophy (Baloh et al., 2007). Because of the vast length of axons in spinal motor and sensory neurons, efficient trafficking of mitochondria is required to meet the bioenergetic demand and maintain calcium homeostasis in the distal projections of the cell. As a result, this suggests a length-dependent axon degeneration pathology where distal regions of the axon are more susceptible to neuropathies than regions proximal to the cell body. The mechanism involved in the selective and progressive axon degeneration in CMT2A remains unclear since mfn2 is a ubiquitously expressed protein (Rojo et al., 2002).

A novel function of mfn2 appears to be in mitochondrial trafficking, distinct from its role in mitochondrial fusion. Expression of mutant mfn2 in dorsal root ganglion neurons disrupted the localization of mitochondria and resulted in aggregation of mitochondria in proximal regions, a consequence of decreased and delayed mitochondria trafficking to the periphery and is consistent with mitochondrial distribution in CMT2A patients (Baloh et al., 2007). This transport defect was independent of cytosolic ATP levels, which are essential for molecular motor activity. There was also normal membrane potential and oxygen consumption levels, indicative of proper mitochondrial function. This provides strong evidence for the importance of mitochondrial trafficking, as dysfunction in trafficking is one of the events that results in the pathology of CMT2A without any apparent effects on mitochondrial function.

It is hypothesized that mfn2 may affect mitochondrial trafficking by playing a regulatory role at the motor complex level. Mfn2 regulates mitochondrial motility by mediating the attachment of mitochondria to microtubules via its interactions with the
miro and milton proteins without being bound to kinesin-1. However, the mechanism is not fully understood (Misko et al., 2010).

Interestingly, the axonal degeneration observed in mfn2 disease mutants was due to loss of proper axonal mitochondrial distribution and not disruption of transport or decreased mitochondrial function (Misko et al., 2012). Blocking of mitochondrial movement by overexpressing syntaphilin, a protein that anchors mitochondria to microtubules without affecting fusion, was not sufficient to induce axonal degeneration (Kang et al., 2008; Lao et al., 2000; Misko et al., 2012). Conversely, loss of fusion activity alone is not solely responsible for axonal degeneration as seen in fibroblasts and HeLa cells expressing a mutant allele of OPA1, the second protein essential for fusion; OPA1 mutations did not interfere with mitochondrial motility, and there was no peripheral axonal degeneration observed (Chen et al., 2005; Olichon et al., 2007).

1.4.3. Movement and Mitophagy Dysfunction

Recent evidence has shown that PINK1 forms a complex with miro and milton proteins: both are associated with anterograde mitochondrial trafficking (Weihofen et al., 2009). This observation suggests PINK1’s involvement in mitochondrial movement. Wang et al. showed that overexpression of PINK1 and/or parkin in rat hippocampal neurons resulted in mitochondrial arrest (Wang et al., 2011). PINK1 requires the presence of parkin, which functions downstream of PINK1, to facilitate this arrest. Interestingly, the mitochondrial targeting sequence of PINK1 is not necessary for this arrest. Following depolarization or uncoupling, PINK1 is imported into the inner mitochondrial membrane and degradation by PARL is disrupted (Youle and van der Bliek, 2012). PINK1 accumulates on the outer mitochondrial membrane, which is indicative of damaged mitochondria. PINK1 phosphorylates miro and recruits parkin. Phosphorylated miro consequently undergoes a parkin-dependent degradation via the UPS. The working hypothesis is that PINK1 and parkin mediate the arrest of damaged mitochondria, isolating them from the rest of the cell prior to their clearance via mitophagy, thus preventing the potential release of pro-apoptotic factors that lead to cellular death, and the accumulation and/or proliferation of damaged mitochondria and mtDNA. This arrest may also serve to prevent fusion of damaged mitochondria to healthy mitochondria as immobile or depolarized mitochondria have a lower probability
of undergoing fusion (Twig et al., 2008; Twig et al., 2010). Using post mortem brain cells from PD patients, it was demonstrated that there was a deficient mitophagy system which lead to an accumulation of autophagosomes at the site of formation (Arduino et al., 2012). This accumulation of autophagosomes was due to the disruption of microtubule based trafficking from the site of formation towards the lysosome to promote fusing between the two organelles and degradation of the vesicle contents. Therefore autophagosome clearance from the periphery of the cell towards lysosomes in the soma is an important process for maintaining cellular health.

PINK1 and parkin may also be required for quality control of mitochondria, a key process that is impaired in patients with the familial forms of PD (Clark et al., 2006; Deng et al., 2008a; Exner et al., 2007; Yang et al., 2008). Elimination of their quality control role leads to the accumulation of damaged mitochondria and subsequent neurodegeneration. Yet, in conjunction with reports that PINK1 may facilitate fission as previously mentioned, PINK1 may mediate the isolation of damaged or misfolded mitochondria via fission followed by the arrest of the unhealthy mitochondrion via the parkin-dependent degradation of miro and subsequent clearance by mitophagy. However, this hypothesis fails to account for the selective degeneration of only dopaminergic neurons in the PD pathophysiology.

The primary mitochondrial functions of ATP generation, calcium buffering and ROS generation have long been known to play an integral role in the proper functions of the nervous system. However, we are becoming increasingly aware that mitochondrial dynamics are also critical for neuronal health and function. It is widely accepted that mitochondrial morphology is regulated by the action of fission and fusion protects. However, it is still unclear how or if mitochondrial morphology can affect mitochondrial function in the cell and consequently influence cellular integrity. Mitochondrial dynamics and its dysfunction are implicated in numerous neurodegenerative diseases (Sheng and Cai, 2012). However, it remains unclear whether dysfunction is a consequence of the disease or directly casual. Particularly, it is unclear whether mitochondrial form affects mitochondrial function or vice versa.
1.5. Aims and Objectives

In this study we investigated the impact of changing mitochondrial motility on mitochondrial morphology and function. Specifically, we overexpressed a dominant negative form of the kinesin-1 (kif5c tail) molecular motor in primary cortical rat neurons and astrocytes then monitored mitochondrial dynamics in real time. Action of kif5c tail on mitochondrial movement operates by competitive inhibition and/or auto-inhibition of endogenous kif5 function (Cross and Scholey, 1999). Primary cortical neurons were used due to their predominant role in various neurodegenerative diseases as described above; however, astrocytes were used as a supplemental because of their flat morphology, which facilitates quantitative measurement of mitochondrial dynamics. We hypothesized that disruption of kif5c mediated movement affects mitochondrial dynamics (shape, fusion, fission, motility) as well as mitochondrial function. Finally, to determine the impact of movement-impaired mitochondria on susceptibility to cellular stressors, we transfected neurons with kif5c tail and then challenged them with a glutamate insult to assess survival rate.

I hypothesised that impairment of kif5c mediated mitochondrial movement would disrupt mitochondrial form and result in mitochondrial dysfunction in primary cortical neurons. Based on the overall hypothesis of the project, my specific objectives and their respective hypotheses are as following:

Objective 1: Examine the effects of disrupted kif5 mediated mitochondrial movement on mitochondrial morphology

Hypothesis 1: Disruption of kinesin-1 mediated mitochondrial movement will reduce mitochondrial length resulting in a change in mitochondrial morphology

Objective 2: Analyze mitochondrial functions in neurons with impaired mitochondrial movement, particularly to examine the effects on cellular ATP and ROS levels

Hypothesis 2: Impaired mitochondrial trafficking will result reduced ATP levels and increased ROS levels in primary cortical neurons
Objective 3: Determine the impact of impaired mitochondrial trafficking on cellular health and survival following glutamate neurotoxicity

Hypothesis 3: Disrupted mitochondrial trafficking will exacerbate neuronal injury after excitotoxic stress
2. Materials and Methods

All chemicals were obtained from Life Technologies (Carlsbad, CA, USA) unless otherwise indicated.

2.1. Cell Culture

Primary cortical neuron-glia cocultures were prepared from embryos of time-pregnant Sprague-Dawley rats (St-Constant, Quebec, Canada) at gestation day 17 as previously described (Brewer et al., 1993; Brocard et al., 2001) with the following modifications. Briefly, fetuses were removed from the uterus, decapitated and the brains were dissected out and placed in ice cold dissection buffer (Ca\(^{2+}\), Mg\(^{2+}\) - free HEPES Buffered Sterile Saline (HBSS: 5.4mM KCl, 0.44mM KH\(_2\)PO\(_4\), 137mM NaCl, 0.63mM Na\(_2\)HPO\(_4\), 10mM Hapes, 5.6mM Glucose) (Sigma-Aldrich, St-Louis, MO, USA), 0.5% penicillin/streptomycin). Cortices were excised and cleaned of the pia mater layer. Cortical tissue was minced, then trypsinized in 0.0375% trypsin-EDTA for 30 minutes at 37°C.

Following trypsinization, 15ml of Neurobasal media supplemented with B-27 containing antioxidants (NB+AO) (200μM glutamax, 0.25g/ml Albumax and 100units/mL penicillin/ 100μg/mL streptomycin) was added and the tissue was further dissociated by trituration using a 10ml serological pipette. The resulting suspension was then filtered through a 70μm nylon mesh cell strainer (Falcon, Bedford, MA, USA) and centrifuged (10 minutes, 340Xg at 4°C). The supernatant was carefully decanted and the pellet was re-suspended in 5ml of NB+AO and treated with 1mg/ml DNAse I (Roche, Indianapolis, IN, USA) for 5 minutes at 37°C. The cell suspension volume was increased to 20mL and centrifuged as described above. The supernatant was removed and the resulting cell pellet was resuspended in 5mL of NB+AO for cell counting. Cell viability and density were determined by exclusion of trypan blue stain and hemacytometer (Hausser...
Scientific, Harsham, PA, USA) counts. The cell suspension was then was diluted and seeded onto 18mm/1D round glass coverslips (Fisher Scientific, Ottawa, Ontario, Canada) in 12 well culture plates (Corning, Corning, NY, USA) at a density of 3X10⁵ cell/well in NB+AO growth media and incubated at 37°C in 95% humidified air/5% CO₂. Prior to cultures, glass coverslips were inserted into the 12-well plates and coated with 0.04mg/ml Poly-D-Lysine (PDL, Sigma-Aldrich, St-Louis, MO, USA) for 2 hours at ambient temperature. PDL was aspirated and culture plates were dried before seeding.

After culture, growth media was completely changed at DIV4 (days in vitro) and replaced with NB-AO (Neurobasal media supplemented with B-27 minus antioxidants). Cultures were maintained by feeding cells at DIV8, 11 and 14; 250μl of media was removed from each well and replaced with 300μl of fresh NB-AO growth media. All experiments on neurons were conducted between DIV11-18 to ensure maturation and completion of glutamatergic synaptic development (Chang and Reynolds, 2006a; Rintoul et al., 2003). Conditioned media removed during feedings was sterile-filtered through 0.22μm syringe-filters and stored at 4°C to be utilised in the neuronal transfection protocol described below.

Primary cultures of rat cortical astrocytes were prepared from post-neonatal day 1 rats according to established protocol (Kaech and Banker, 2006). Astrocytes were grown in Complete Astrocyte Media (CAM: 83.3% BME, 15% FBS, 100units/mL penicillin/100μg/mL streptomycin, 20mM glucose, 200μM glutamax). Astrocytes were preserved in liquid nitrogen using cell freezing media (50% FBS, 40% CAM, 10% DMSO) until needed. Cells were then thawed and grown in sterile 75cm² tissue culture flasks (Falcon, Bedford, MA, USA). Once confluence reached approximately 90%, the astrocyte content was enriched by mechanical shock to detach less adherent cell types such as oligodendrocytes and neurons. Cells for experimentation were seeded in 12-well plates at 1ml/well of cell suspension. The astrocytes were feed twice a week by replacing 250μl of media from each well with 300μl of fresh CAM. Astrocyte cultures were propagated for up to 6 passages before being discarded.

All protocols involving animals were in accordance with the Canadian Council on Animal Care (CCAC) and were approved by the Simon Fraser University Animal Care Committee (SFU UACC).
2.2. DNA Plasmid Constructs

Mitochondria were visualized in vitro using an enhanced yellow fluorescent protein (mito-eYFP) provided by Dr. Roger Y. Tsien (University of California, San Diego, USA). Mt-eYFP contains the targeting sequence for complex IV of the electron transport chain, cytochrome c oxidase, which allows visualization of the mitochondrial matrix using the mammalian expression vector pCDNA3.

Disruption of mitochondrial movement was achieved by overexpressing a fluorescent-tagged kif5c-tail construct generously provided by Dr. Yoshiyuki Konishi (Hamamatsu University School of Medicine, Japan). The plasmid has only the tail domain of the kinesin-1 (kif5) which contains the mitochondrial binding sequence. Constitutive expression of kif5c tail is capable of competitive and/or autoinhibition of all kinesin-1 subtypes (kif5a, b, and c) (Cross and Scholey, 1999; Konishi and Setou, 2009). Control cells were transfected with a cytosolic expressed RFP (n-fusion RFP). Cytosolic RFP was used to control for any effects due to overexpression of large foreign fluorescent proteins as well as possible toxicity to neurons and astrocytes arising from the transfection protocol described below.

To determine the effect of kif5c tail on other cargoes transported by kif5, we fluorescently tagged lysosomes and α-tubulin subunits using LAMP1-GFP (Addgene.org) and α-tubulin GFP (Addgene.org), respectively, in neurons overexpressing kif5c tail. Subcellular distribution patterns of each organelle were examined. We also tagged a dynein driven cargo, autophagosomes, using LC3-GFP (Addgene.org) to examine the effect on retrograde trafficking (Kimura et al., 2008; Longatti and Tooze, 2009; Yamamoto et al., 2010)

2.3. Transfection Protocol

Transfections on primary cortical neurons and astrocytes were done using Lipofectamine 2000 (LF 2000) protocol (Dalby et al., 2004). For each coverslip, 1μg of the DNA plasmid of interest and 1μl LF2000 were diluted in separate tubes containing 50μl unsupplemented NB, and incubated for 5 minutes at room temperature. Both
mixtures were then gently combined to make a transfection cocktail and left to incubate for an additional 20 minutes at room temperature to allow plasmid-LF complexes to form. Prior to transfection, the media volume in each well was reduced to 400μl, and then 100μl of the transfection cocktail was added. The ratio of LF2000: DNA used was 1μl:2μg during a dual transfection and 1μl:1μg for single transfections for every 100μl of transfection mix. Cells were incubated in the transfection cocktail at 37°C in 95% humidified air/5% CO₂ for 6-8hrs before termination of transfection. The transfection mix was aspirated and replaced with 1ml/well of conditioned NB-AO. Cells were incubated for an additional 12-16 hours to allow for transgene expression before fluorescence imaging. Transfection efficiency is typically 1-3% using lipofectamine-mediated transfection in primary neuron cultures (Funke et al., 2011; Rintoul et al., 2003).

Astrocyte transfections were performed when cells were approximately 75% confluent, using a similar LF transfection protocol. However, instead of using unsupplemented NB, Basal Media Eagle (BME) was used during incubations. Also, transfection reactions were terminated when the transfection mix was replaced with Complete Astrocyte Media rather than conditioned NB-AO.

### 2.4. Fluorescence Imaging

Live cell imaging and data acquisition was done using Simple PCI software (Compix Inc. Cranberry, PA) on a Nikon Eclipse TE2000E2 inverted fluorescence microscope and a Lambda-LS Xenon Arc lamp light source. All images were taken with a 40X 0.6 NA (Plan Fluor ELWD) objective lens with a binning of 2 and at ambient temperature unless stated otherwise. Each coverslip was imaged in a magnetic microincubation chamber (Quorum Technologies) bath in 500μl of HBSS (HEPES Buffered Saline Solution) adjusted to pH 7.4 and containing: 137mM NaCl, 5.4mM KCl, 10mM NaHCO₃, 20mM HEPES, 5.5mM glucose, 0.6mM KH₂PO₄, 0.6mM Na₂HPO₄, 1.4mM CaCl₂, and 0.9mM MgSO₄. The excitation/emission wavelengths used were 492nm/536nm for YFP and GFP 580nm/630nm for RFP.
2.5. Mitochondrial Dynamics Analysis

2.5.1. Mitochondria Morphology and Length

Mitochondrial length was measured using two methods: manual and semi-automated traces. For manual traces, each discrete mitochondrion within a 255x255 pixel sub-field was measured using a tracing function of Simple PCI software. For astrocytes, 90-120 mitochondria were analysed; however, in the case of neurons, all visible mitochondria in the field were measured. The perinuclear region in astrocytes and the cell body in neurons were avoided during analysis due to mitochondrial clustering and overlapping. Semi-automated traces were performed using macros of the Image Processing and Analysis (IPA) module of SimplePCI once micrographs were acquired. Mitochondria were enhanced and identified based on set criteria; to exclude background and autofluorescent debris, mitochondria were identified based on a minimum fluorescence intensity. In order to avoid analysis of the perinuclear region which was filled with mitochondrial clusters, the entire soma of the neurons were excluded based on their relatively large area compared to an individual mitochondrion. Mitochondrial length was then computed and expressed as pixels. Known calibration factors were used to convert pixels to actual length.

To evaluate the correlation between kif5c tail expression and its effect on mitochondrial length further, all micrographs were taken at an exposure of 0.2sec. Mitochondrial length was determined in parallel with kif5c tail expression (reflected by the fluorescence intensity), and then the Pearson’s moment correlation coefficient (r) was used as a measure of the dependence between the two variables.

2.5.2. Mitochondrial Occupancy and Density

To assess mitochondrial occupancy inside neuronal processes, a single process was chosen and the total length of all mitochondria within the process was divided by the length of the process. This value was expressed as percentage process filling. Mitochondrial density within a given process was determined by dividing the number of mitochondria by the length of the process. Density measurements were expressed as mitochondrial count per 100 microns.
2.5.3. Mitochondrial Movement

Mitochondrial movement was monitored in real time at 37°C for neurons and at ambient temperature for astrocytes. Time lapse videos were generated from 15 successive images taken at 10 second intervals. Movement was analysed using Simple PCI and an Excel macro developed by Dr. Rintoul as previously described (Rintoul et al., 2003). Based on edge detection, mitochondria were identified (see below) and monitored for changes in fluorescence between each successive frame. This is referred to as bulk movement and is a non-directional measure, including anterograde and retrograde movement as well as oscillatory motion (also referred to as “wiggling”). Before processing each movie, a fluorescence threshold was applied to identify mitochondrial pixels and distinguish them from non-mitochondrial (i.e. background) fluorescence. Mitochondrial intensity transitions from bright-to-dark or dark-to-bright in individual pixels were then registered as “movement events”. These were normalized by dividing movement events by the total number of pixels occupied by mitochondria, and the data plotted as movement events per pixel.

To determine the effects of differing kif5c tail levels of expression on bulk movement in neurons, time lapse movie were generated at exposures of 0.2sec representative of high kif5c tail expression and at 0.4sec or 0.8sec representing low kif5c tail expression.

Velocity of mitochondria in neurons was also determined using the Motion Trafficking Analysis (MTA) module of Simple PCI. Mitochondria were identified with the IPA module; using edge detection algorithms, a replicate masked image was created from the original micrographs based on the fluorescent contrast between mitochondria and background. Once each mitochondrion was identified, the individual object trajectory was tracked between successive frames based on their area, centre of gravity and position on x/y coordinates. Both straightline and curvilinear velocities were computed.

2.6. Reactive Oxygen Species Measurements

Cytosolic ROS levels were determined using a ROS-sensitive fluorescent indicator, ro-GFP (generously provided by Dr J. Remington, University of Oregon, USA)
(Hanson et al., 2004). The probes are GFP mutants containing two cysteine substitutions of surface-exposed residues at positions Q204 and S147 of the β-strand (fig. 2). Ro-GFP operates on the principle of excitation ratiometry where the fluorophore has two different excitation maxima, 380nm and 492nm, whose peak amplitude depends on the oxidation state. In an oxidizing environment, the cysteine residues form tight disulphide bridges which cause a strain on the polypeptide backbone resulting in a change in the spectral properties of the GFP. The formation of disulphide bridges promotes the protonation of the fluorophore (Dooley et al., 2004). This results in a shift of the peak excitation amplitude at 492nm to 380nm wavelength.

To examine cytosolic ROS levels, the redox-sensitive probe ro-GFP was used. Based on the ratiometric properties of ro-GFP, imaging was done by alternately exciting the probe at 380nm and 492nm wavelengths. Relative ROS level were determined by measuring the ratio of fluorescence intensity emission at 535nm following alternate excitation ratio at 380nm and 492nm in cortical neurons transfected with the probe. A binning of 4 was used for the probe.

To establish the range of sensitivity of ro-GFP in primary cortical neurons, a continuous time course assessment was performed following application of a strong oxidant and reductant. Time lapse images were generated every 60 seconds over approximately 90 minutes. Neurons transiently expressing only ro-GFP were perfused at a rate of 1ml/min with room temperature HBSS for approximately 15 minutes to establish a stable baseline intensity reading. To introduce an oxidative stressor, cells were perfused with 100μm H₂O₂ for about 25 minutes. Then cells were perfused with a strong reductant 1mM dithiothreitol (DTT), for another 25 minutes. To confirm the reversibility of the probe, cells were washed out with HBSS for 25 minutes to determine if baseline levels could be re-established following DTT perfusion. For each cell, fluorescence intensity was recorded from 3 regions of interest selected within the cell. Background correction was performed by subtracting the intensity measurements (at 380nm and 492nm excitation wavelengths) from a nearby field that was devoid of cells before calculating excitation ratios.
To assess the effect of overexpression of kif5c tail on ROS generation in cortical neurons, cells were dually transfected with kif5c tail and ro-GFP as per the LF2000 protocol. Mean ro-GFP ratios were then examined as described above.

Figure 2. **Structural features of the ROS-sensitive probe, ro-GFP**

(a) Surface exposed residues on the Green Fluorescent Protein (GFP) at positions S147/Q204 were substituted with cysteine (b) These substitutions result in the formation of disulphide bonds in an oxidizing environment and shift the excitation maxima of the chromophore from 492nm to 380nm. Note that the variant of the probe employed in this exhibits cytoplasmic expression. Used with permission from the author.

2.7. **ATP Measurements**

Real time measurement of cellular ATP levels was done using “Adenosine Triphosphate indicator based on ε subunit for analytic measurement” (ATeam) biosensors based on fluorescence resonance energy transfer (FRET) principles (Imamura et al., 2009). ATeam probes were generously provided by Dr. H. Imamura.
The ATeam probe consists of the ε subunit from *Bacillus subtilis* F₀F₁-ATP synthase sandwiched between mseCFP (donor fluorophore) and mVenus (acceptor fluorophore), variants of CFP and YFP, respectively (fig. 3). The ε subunit binds to ATP without undergoing hydrolysis and has a higher binding specificity for ATP over other nucleotides (Wang et al., 2006). In an ATP-free state the ε subunit is extended and flexible between the two fluorochromes and will result in low FRET efficiency. However, when in an ATP-bound state, the ε subunit undergoes a conformational change that causes a retraction, shortening the distance between YFP and CFP and resulting in an approximate 2 fold increase of FRET efficiency (Imamura et al., 2009). These conformational changes are reversible (Santel and Fuller, 2001).

Relative cellular ATP levels were measured using the ATP-FRET based probes ATeam. Neurons were transfected with the probe and excited at 436nm. Fluorescence intensity for YFP (535nm) and CFP (465nm) emissions were recorded. Following similar background subtraction methods previously used, the emission intensity ratio (535nm/465nm) was calculated as a measure of the relative level of ATP in the cells. To validate the range of sensitivity for the probe in neurons, cells were perfused with 1mM potassium cyanide (KCN), an inhibitor of oxidative phosphorylation at a rate of 1ml/min for 90minutes.

Finally, to determine the effect of overexpression of kif5c tail on ATP generation, neurons were cotransfected with kif5c-RFP and ATeam. Three regions of interest within the cytoplasm were selected and measured for mean emission ratios 535nm/465nm and compared to control cells which were cotransfected with n-fusion RFP and ATeam.
2.8. Cell Survival Assay (Excitotoxicity)

Single transfections were performed on neurons using either kif5c tail or n-fusion (control). Cells were allowed to express plasmids for 12-16hrs before neurotoxic treatment. To induce excitotoxic stress, each treatment group (Kif5c tail and control) was bathed in 50μm glutamate (prepared in HBSS) for 10 minutes at 37°C. As a negative control, cells were incubated in sterile HBSS under the same conditions. This sub-group was used to establish cell counts for 100% survival. Additionally, neurons were treated with 250μm glutamate for 10 minutes at 37°C to ensure global cell death as a positive control. Each coverslip was rinsed in HBSS prior to and after glutamate application.
Cells were then incubated in 1 ml/well of conditioned NB-AO for 24hrs to allow recovery from the stressor before imaging to assess cell viability.

In all experiments, neuronal cell counts along the diameters of both the x and y axes of each coverslip were summed to represent cell count per coverslip. Neurons were identified based on the morphology of kif5c tail or n-fusion RFP positive cells; only those exhibiting uniform expression of each probe through soma and processes were included. We excluded any cells that showed distinct disintegration of the processes from the soma. Neuron survival was determined by expressing the number of kif5c tail-positive cell in each sub-group (50μm or 250μm glutamate) as a proportion of the negative control (HBSS alone). Survival rate was expressed as a percentage. Similar assessments were made for the control group.

2.9. Statistical Analysis

All data were analysed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) and are shown as mean ±S.E.M. Statistical differences were analysed using unpaired student t tests and one-way ANOVA with the Bonferroni post-test. Correlation analysis was performed using the Pearson moment correlation. P-value <0.05 indicated significant differences.
3. Results

For this study, primary cultures of rat cortical neurons and astrocytes were used as a model system to study the effect of disrupted mitochondrial trafficking. Cultured neurons used for experimentation were predominantly pyramidal neurons (fig. 4a). Their unique architecture consists of a triangular soma with extensive arborisations; they typically have one long axon and multiple apical and basal dendrites. Alternately, astrocytes were used based on their advantageous morphology. Cultured primary cortical astrocytes had a distinct flat “fried-egg” topology with stellate morphology as observed with phase contrast imaging which permitted good visualization of mitochondria (fig. 4b).
Figure 4. **Micrograph of model cell systems**

(a) phase contrast image of primary cortical neurons at DIV 11 (days in vitro) seeded at $3 \times 10^5$ cells/ml in NB-AO (b) phase contrast image of cortical astrocytes at 50% confluence maintained in complete astrocyte media.

3.1. **Effects of Kif5c Tail Overexpression on Mitochondrial Dynamics in Astrocytes**

Dual transfection of 80-90% confluent astrocytes with the mitochondrial label, mt-eYFP, and kif5c tail resulted in altered mitochondrial morphology. Cells overexpressing the kif5c tail had punctate (or rounded) mitochondria compared to controls which exhibited elongated mitochondria (fig. 5a-b). The subcellular distribution of mitochondria was also affected. Perinuclear clustering of mitochondria was observed in astrocytes overexpressing kif5c tail when compared to control cells (fig. 5a-b). Mitochondrial length acquired using two comparable methods (see materials and methods for details) indicated a reduction due to overexpression of kif5c tail (fig. 5c). Mean mitochondrial length in control cells was $5.85 \pm 0.24 \mu m$ (n=18 cells) versus cells transfected with kif5c tail at $4.23 \pm 0.18 \mu m$ (n=21 cells), representing a 27.6% decrease. The histograms (fig.
5c) represent mitochondrial length generated using the semi-automated traces. It is also noteworthy that mitochondrial YFP intensity in kif5c tail transfected cells appeared brighter than in control cells.

Bulk movement was used as a measure of the spatiotemporal mitochondrial motility within astrocytes. Interestingly, the overexpression of the dominant negative form of kif5c showed no significant difference in bulk movement compared to the control (fig. 5d). Bulk movement observed in the control was 0.12 ± 0.021 event count/pixel (n=9 cells) compared to kif5c tail 0.10± 0.012 event count/pixel (n=17 cells). Note all imaging for astrocyte mitochondrial dynamics was done at a binning of 1.
3.2. Effects of Kif5c Tail Overexpression on Mitochondrial Dynamics in Cortical Neurons

Disruption of mitochondrial movement was achieved by the exogenous overexpression of kif5c tail in primary cortical neurons. There was uniform distribution of the kif5c tail construct throughout the cells, extending into the neuronal processes with an enhanced expression in cell body (fig. 6b). Overall, kif5c tail was cytoplasmic as expected. Concomitantly, mitochondria were monitored using a mitochondrially targeted
fluorescent YFP, mt-eYFP. Mt-eYFP was evenly dispersed throughout the neuronal processes with a clustering observed in the soma excluding the nuclear region; however, being organelle specific to the mitochondrial matrix, the fluorescence pattern of mt-eYFP had a distinct shape (fig. 6c).

Mitochondrial morphology in cells expressing the kif5c tail protein was punctate compared to the typical elongated appearance seen in control cells (fig. 7b,d). Similar to effects observed in astrocytes, mitochondrial intensity also appeared brighter in neurons when cotransfected with kif5c tail. There was also a change in the subcellular distribution of mitochondria in the soma (fig. 7a,c). The punctate morphology of mitochondria in kif5c tail transfected neurons was associated with the shortening of mitochondria (fig. 8a). Mean mitochondrial length in kif5c tail expressing neurons was $1.93 \pm 0.08 \mu m$ (n=39 cells). Compared to control cells ($2.54 \pm 0.27 \mu m$, n=18 cells), there was a 23.77% significant decrease in mitochondrial length.

To determine whether the detected shortening of mitochondria was due to fragmentation or remodelling was the cause of the shortened mitochondria detected, mitochondrial occupancy and density were determined. Mitochondrial occupancy, which measures the percentage of process length occupied by mitochondria, was significantly decreased (11.89% reduction) in kif5c tail transfected neurons compared to control (fig. 8b). Occupancy dropped from 61.8 $\pm 2.89\%$ (n=18 cells) in controls to 46.91 $\pm 3.25\%$ (n=24 cells) in cells expressing kif5c tail. However, mitochondrial density measuring the number of mitochondria per unit process length showed no significant differences between control and kif5c tail neurons (fig. 8c). Density in the control was 17.85 $\pm 1.04$ mitochondria/100$\mu m$ (n=18 cells) versus 19.37 $\pm 0.75$ mitochondria/100$\mu m$ (n=24 cells) measured in kif5c tail transfected neurons. Collectively this implies that mitochondrial distribution may be altered as less space within neuronal processes is being occupied by mitochondria.

Our results suggest decreased mitochondrial length is a result of remodelling and not increased mitochondrial fission. However, we cannot completely discount the possibility that upregulation of both fusion and fission maintains the number of mitochondria in the neurons.
Figure 6.  Micrograph of a typical primary cortical neuron dual transfected with kif5c tail-RFP and mitochondrially targeted YFP

(a) Neurons between DIV 11-15 were transfected using 1ug/µl of each DNA plasmid. (b) Kif5c tail (pseudo colored red) shows diffuse cytoplasmic distribution in the cells (c) mitochondria labeled with mito-eYFP (pseudo colored green) also appear evenly distributed throughout the neuronal processes. There was also an accumulation of mitochondria in the soma excluding the nucleus. Scale bars 30µm
Figure 7.  *kif5c tail overexpression changes mitochondrial morphology in primary cortical neurons.*

(a,b) Micrographs displaying mitochondrial labelling by mt-eYFP show change to mitochondrial shape in control neurons compared to neurons overexpressing kif5c tail.  (c,d) Mitochondrial shape is altered from the typical elongated tubular shape to a more punctate shape suggesting possible fragmentation.
Figure 8. Effects of overexpressed kif5c tail on mitochondrial length and percent process filling in primary cortical neurons

(a) There was a 23.77% decrease in the mitochondrial length for neurons overexpressing kif5c tail (kif5c tail 1.93 ± 0.08μm, n=39cells; control 2.54 ±0.27μm, n=18cells) (b) Although there was a reduction in the mitochondrial process filling, no significant differences in the mitochondrial count/100micron was observed (mitochondrial count/100micron: control 17.85 ±1.04, n=18cells; kif5c tail 19.37 ±0.75, n=24cells) suggesting that reduction of mitochondrial length was predominantly due to remodelling not fragmentation. (process filling/occupancy: control 61.8 ±2.89% n=18cells; kif5c tail 46.91 ±3.25%, n=24) p-value <0.05

3.3. Inverse Correlation between Kif5c Tail Expression and Mitochondrial Length

Visual inspection of mitochondrial morphology revealed a recurrent pattern, which suggests that reduction of mitochondrial length may depend on the level of kif5c tail expression in cortical neurons. Neurons expressing high levels of kif5c tail, indicated by a strong red fluorescence signal, exhibited a discrete punctate mitochondrial shape as described above (high kif5c tail 1.78±0.09μm, n=26 cells) (fig. 9a-b). On the other hand, there was a striking similarity in mitochondrial morphology between control cells
and neurons expressing low levels of kif5c tail; mitochondria shape was predominantly 
elongated similar to control cells (fig. 9c-d). Mitochondrial length was comparable 
between low kif5c tail expressing neurons and controls (low kif5c tail 2.27 ± 0.14μm, 
n=24cells; control 2.54±0.27μm, n=18cells) (fig. 9e). Furthermore, this suggests that 
effects of kif5c tail on mitochondrial length we reported above when all kif5c tail 
transfected neurons were grouped together (fig. 8a) may be an underestimation of the 
true effects. It is important to note that all micrographs were taken at the same exposure 
(0.2sec). To demonstrate the correlation between kif5c tail expression and mitochondrial 
length, we calculated the Pearson’s moment correlation coefficient (r). Our data 
indicated a significant inverse correlation exhibited between kif5c tail expression and 
mitochondrial length with r= -.502 (fig.9f, n=41 pairs).

In summary, these data suggests that reduction in mitochondrial length are 
correlated with the level of kif5c tail expressed in primary cortical neurons, and further 
supports our finding that kif5c has a role in regulating mitochondrial morphology.
Figure 9. Extent of alteration to mitochondrial morphology appears dependent on levels of kif5c tail expression in neurons

(a-b) Shows a neuron with punctate mitochondria expressing high levels of kif5c tail compared with figure (c-d) showing a neuron with elongated mitochondria expressing low levels of kif5c tail. (e) Neurons expressing low levels of kif5c tail had comparable mitochondrial length typical of control cells while those expressing higher levels of kif5c tail had significantly shorter mitochondria (mitochondrial length: all kif5c tail 1.78±0.09μm, n=26 cells; low kif5c tail 2.27±0.14μm, n=24; control 2.54±0.27μm, n=18) (f) A summary of mitochondrial length show an inverse relationship between the mean mitochondrial length and the level of exogenous kif5c tail expressed in the neurons (r=-0.503), p-value <0.05.
3.4. Overexpression of Kif5c Tail Impairs Mitochondrial Movement

Sequential time lapse images were generated to analyse the effect of overexpression of kif5c tail on mitochondrial trafficking. Fifteen fields were collected with 10 second intervals. Fig 10a shows a representative region of interest in cortical neuron overexpressing kif5c tail. The majority of mitochondria were stationary throughout the movie (shown with the green arrow). Interestingly, we also observed a pronounced oscillatory motion compared to directed movement by the mitochondria in kif5c tail expressing neurons. Kif5c tail did not abolish total mitochondrial movement; in some instances, we observed highly reduced movement. The red arrow shows a fusion event in progress that failed to reach completion before the end of the time course. In comparison, mitochondrial movement in control cells was highly dynamic (fig. 10b). Between fields 1 and 5, two individual fission events appear to have undergone completion (red and yellow arrows). By field 10, mitochondria were moving out of the ROI (yellow arrow) and a series of fission, fusion and trafficking events are apparent (blue arrow).

Two approaches were used to quantify mitochondrial trafficking: 1) comparison of bulk movement, which measures the “movement event” generated from 15 sequential images (see materials and methods for details); this approach does not distinguish between direct, lateral and/or oscillatory movement, and 2) comparison of straightline and curvilinear velocities of the entire neuron using the MTA module of Simple PCI.

Bulk movement was decreased in kif5c tail expressing neurons (fig. 10c). There was a 15.7% reduction in kif5c tail expressing neurons (0.18±0.008events/pixel, n=29cells) compared to control cells (0.21±0.014events/pixels, n=20cells). To decipher whether there was a kif5c tail expression dependent effect on bulk movement, we measured bulk movement at 2 different levels of kif5c tail expression. Kif5c tail expression levels were categorized based on the exposure time used to capture time lapse images. High kif5c tail expression was collected using an exposure of 0.2sec with an average intensity of 150 ±23.1 fluorescence intensity (F.I.) units (n=14cells), whereas low kif5c tail expression was collected at 0.4 and 0.8sec exposures with an average intensity of 28.03 ±2.25 F.I. units (n=15cells) (data not shown).
We found bulk movement in neurons expressing low levels of kif5c tail was comparable to control cells (low kif5c tail = 0.219±0.012 events/pixels, n=17 cells; control = 0.218 ±0.016 events/pixels, n=17 cells) (fig. 10d). There were also more oscillatory movement of mitochondria in high kif5c tail expressing cells compared to low kif5c tail expressing neurons. Finally, as expected there was a significant difference between neurons expressing high versus low levels of kif5c tail (high kif5c tail = 0.18±0.009 events/pixel n=23 cells; low kif5c tail = 0.219±0.012 events/pixels, n=17 cells).

Overexpression of kif5c tail did not affect curvilinear velocity of mitochondria within the neurons (kif5c tail = 2.28±0.19 microns/sec, n=56 cells; control = 1.92±0.23 microns/sec, n=37 cells) (fig. 10e & Table 1). We did not distinguish between low or high kif5c tail expression levels when calculating velocity parameters. There was a significant decrease in mitochondrial straight line velocity in kif5c tail neurons. Straight line velocity dropped from 0.69±0.07 microns/sec (n=37 cells) in control cells to 1.06±0.14 microns/sec (n=56 cells) in neurons overexpressing kif5c tail. Interestingly, there was a higher percent of mitochondria in motion in kif5c tail cells (Table 1).
Figure 10. Overexpression of exogenous kif5c tail alters mitochondrial movement in primary cortical neurons.
(a) Time course of mitochondrial movement in neurons transfected with kif5c tail show highly reduced movement. Note the red and green arrows show points of reference for minimal and no movement of each mitochondrion in the field respectively. (b) In control, mitochondrial movement is observed; all arrows indicate the movement of each respective mitochondrion. Note that the mitochondrion tracked with the yellow arrow moves out of the field of view after field 10. Scale bar 15 um (c) Mitochondrial movement expressed as “event count/pixel” of neurons with overexpressed kif5c tail showed a decrease compared to control (kif5c tail 0.18±0.008 events/pixel, n=29 cells; control 0.21±0.014 events/pixels, n=20 cells) (d) The severity of impaired movement was dependent on the level of expression of kif5c tail in the neurons (high kif5c tail images where shot at an exposure of 0.2 sec with mean kif5c tail intensity 150±23.1 F.I. units, n=14 cells; low kif5c tail exposure 0.4 sec and 0.8 sec, kif5c tail intensity 28.03±2.25 F.I. units, n=15 cells). Note that movement in cells expressing low kif5c tail level was comparable to control neurons (high kif5c tail 0.176±0.009 events/pixels, n=23 cells; low kif5c tail 0.219±0.012 events/pixels, n=17 cells; control = 0.218±0.016 events/pixels, n=17 cells) (e) Kif5c tail reduced the straightline velocity of mitochondria (straight line velocity: control 1.06±0.14 microns/sec, n=37 cells; kif5c tail 0.69±0.07 microns/sec, n=56 cells) without affecting curvilinear velocity in neurons (kif5c tail =2.28±0.19 microns/sec, n=56 cells; control= 1.92±0.23 microns/sec, n=37 cells) , p-value<0.05.

To confirm that mitochondrial trafficking impairment was not a result of cytoskeletal depolymerization, we conducted a single transfection with kif5c tail to assess the architecture/morphology of the neurons. Our results suggest that microtubule organization and integrity are not compromised (fig. 11). Neuronal processes in kif5c tail cells extended from the soma and have the typical tubular morphology with numerous reticulations. Dendritic spines were also visible. Collectively, these results suggest that overexpression of kif5c tail impairs mitochondrial movement without compromising microtubule integrity (see below for additional information of tubulin distribution in neurons).
<table>
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<td>1.92±0.23</td>
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</tbody>
</table>

*Table 1. Summary of mitochondrial movement parameters in primary cortical neurons*

All values were computed using Simple PCI Motion Trafficking Analysis (MTA) module.* indicates statistical differences between cells overexpressing kif5c tail versus control where p<0.05.
3.5. Effect of Kif5c Tail on Trafficking of Other Cargo

To evaluate whether kif5c tail overexpression had a specific effect on mitochondrial transport or a global effect on intracellular movement, we performed a qualitative analysis on the subcellular distribution of other kif5c cargo (α-tubulin subunits and lysosomes) and a dynein mediated cargo (autophagosomes). Co-transfection with kif5c tail and the respective cargo marker was performed. Overall, distribution patterns of α-tubulin and autophagosome labelled with α-tubulin-GFP and LC3-GFP respectively remained unaltered despite the high levels of kif5c tail observed in primary cortical neurons. α-tubulin remains equally distributed throughout neuronal processes (fig. 12c, f). Similarly, LC3-GFP distribution was evenly dispersed throughout the cell, appearing cytoplasmic (fig 14c, f).
Figure 12. **Effect of overexpression of kif5c tail on the localization of alpha-tubulin.**

(Panels a,b,c) There was no change in the distribution pattern of alpha-tubulin (pseudo-coloured green) in control cell compared to cortical neurons overexpressing endogenous kif5c tail (panels d,e,f). Scale bar 30μm.

In control cells, lysosomes appeared as punctate organelles that were uniformly distributed throughout the neuron, including the distal processes, when labelled with LAMP1-GFP (fig. 13f). There was an accumulation of lysosomes in the soma; however they were excluded from the nucleus. In neurons overexpressing kif5c tail, lysosomes appeared to display reduced aggregation of puncta in the soma compared to control neurons (fig. 13c). Also the uniform distribution in the processes showed aberrations; there were minimal lysosomes detected in a majority of the processes, and when visible, they accumulated closer to the soma compared to the cell periphery.

Collectively, our data suggest that although kif5c tail does not have a global effect on intracellular trafficking, it alters lysosome transport and distribution.
Figure 13. Effect of overexpression of kif5c tail on the localization of lysosomes.

Dispersion of lysosomes throughout primary cortical neurons appears altered by the overexpression of kif5c tail. Scale bar 30µm
Figure 14. Effect of kif5c tail overexpression on the localization of dynein transported cargo in primary cortical neurons.

Panels (a) and (c) show the distribution of autophagosome using an LC3 marker (pseudo-coloured green), there was no observable difference in LC3 distribution neurons in control cells compared to neurons overexpressing kif5c tail (panel d,f). Scale bar 30μm

3.6. Characterization of ATeam in Primary Cortical Neurons

Intracellular ATP levels were measured using an ATP sensitive FRET based probe, ATeam, constructed by Imamura et al. (Imamura et al., 2009). We determined the relative levels of ATP in the cell based on ATP interaction with the ε subunit from Bacillus subtilis F$_0$F$_1$-ATP synthase. This interaction results in increased FRET efficiency from the donor fluorophore, CFP, to the acceptor fluorophore, YFP. The version of the probe we utilized allows for measurement of ATP in the millimolar range (Imamura et al., 2009). Transfection of cortical neurons with the ATeam showed uniform distribution throughout the cell that is cytoplasmic; there were no signs of probe aggregation or compartmentalization in the neurons (fig.15a).
To validate the ATeam range of sensitivity, neurons expressing the probe were perfused with 1mM KCN, an inhibitor of oxidative phosphorylation. Addition of KCN resulted in a moderate fluorescence increase in the CFP channel (465nm) and a large decrease in the YFP channel (535nm) (fig. 15b). Baseline emission ratio (535nm/465nm) declined from 4.07±0.10 (n=4 cells) to 1.68±0.12 after 30min exposure to 1mM KCN, indicating depletion of cytoplasmic ATP levels. Previous *in vitro* studies have also shown ATeam sensitivity to a 2.5 fold increase in ATP levels in HeLa cells (Imamura et al., 2009).

**Figure 15.** Characterization of sensitivity range of FRET-based ATP sensitive probes (ATeam) in primary cortical neurons.

(a) Single transfection of cortical neurons with ATeam probe. (b) Time course of fluorescent intensity showing the quantitative analyses of ATeam sensitivity to the presence of cytoplasmic ATP expressed as 535nm/465nm ratio. A 90 minute perfusion was performed at a rate of 1ml/min. Cells were perfused with HBSS for 20mins to establish baseline readings, thereafter were perfused with 1mM KCN (inhibitor of oxidative phosphorylation).
3.7. Effect of Kif5c Tail Overexpression on ATP Generation

To determine whether ATP generation was affected by the atypical mitochondrial morphology as a result of kif5c tail overexpression, cortical neurons were co-transfected with kif5c tail-RFP and ATeam. Our results indicated that baseline ATP levels were higher in neurons expressing kif5c tail relative to control cell by 5.88% (fig. 16). The emission ratio (535nm/465nm) was 4.32±0.06 (n=41 cells) in kif5c tail neurons versus 4.08±0.06 (n=21 cells) in control cells.

![Figure 16](image)

*Figure 16. Overexpression of kif5c tail increases the relative levels of ATP production in primary cortical neurons.*

Neurons expressing kif5c tail showed an elevation in the relative levels of cytoplasmic ATP (535nm/465nm: 4.32 ±0.06, n=41 cells) compared to control (535nm/465nm: 4.08±0.06, n=21 cells). P-value <0.05

3.8. Characterization of Ro-GFP in Primary Cortical Neurons

ROS generation was analysed using the redox-sensitive probe ro-GFP. It possesses two excitation absorption peaks (380nm and 492nm) that are based on the oxidation state of the molecule. Transfection of neurons with ro-GFP shows that
distribution of the probe is cytoplasmic (fig. 17b). It is evenly distributed throughout the cell with an accumulation in the cell body; however, it was excluded from the nucleus and did not show any signs of compartmentalization.

To examine the range of detection for ro-GFP, we monitored the neurons transiently expressing ro-GFP following perfusion with a strong oxidant, 100μm, H₂O₂ and a strong reductant, 1mM DTT. With addition of H₂O₂, fluorescence intensity at 492nm had a pronounced decrease whereas intensity at 380nm showed only a slight increase; there was a shift in the absorption peak from 492nm to 380nm (fig. 17a). The excitation ratio (380nm/492nm) increased from a baseline of 0.782 ±0.035 (n=3 cells) to 1.1±0.02 (n=3 cells), demonstrating an oxidized environment. Subsequent perfusion with a strong reductant elicited a decrease in 380nm/492nm to 0.576±0.012 (n=3 cells) after about 20 minutes. As expected, there was an approximate 2 fold increase in intensity from the 492nm channel and a corresponding decrease in the 380nm channel. Finally, a wash-out with HBSS reversed 380nm/492nm near baseline levels 0.621±0.31 (n=3 cells). Our data support the ratiometric nature of the probe, as well as its reversibility in detecting ROS levels in primary cortical neurons.
3.9. Effect of Kif5c Tail Overexpression on ROS Generation

Mitochondria are the primary generators of cellular ROS via the electron transport chain; disruption of ROS homeostasis has been implicated in various neurodegenerative diseases (Bindokas et al., 1996). To determine the effect of kif5c tail on ROS generation, neurons were transfected with ro-GFP and kif5c tail. Our data showed a 12.72% decrease in kif5c tail neurons relative to the control, indicative of a
relatively reduced cellular environment (fig. 18). The excitation ratio (380nm/492nm) in kif5c tail cells was 0.71 ±0.02 (n=40 cells) versus control 0.81±0.03 (n=32 cells).

Figure 18. Effect of overexpression of kif5c tail on relative ROS levels in primary cortical neurons

ROS level were significantly reduced by 12.72% in neurons overexpressing kif5c tail compared to control (380nm/492nm: kif5c tail 0.71 ±0.02, n= 40cells; control 0.81±0.03, n=32cells), p-value <0.05

3.10. Effect of Kif5c Tail on Cell Survival after Excitotoxicity

It has been hypothesized that mitochondrial dysfunction might facilitate excitotoxic death by making neurons more susceptible to intracellular glutamate increase (Chen et al., 2005). Because overexpression of kif5c tail appears to affect mitochondrial bioenergetics in our study, we investigated the effect of kif5c tail on the vulnerability of neurons to excitotoxic injury (using 50μm glutamate for 10 mins) after a 24 hour recovery period. We included both positive and negative controls treated with 250μm glutamate and HBSS respectively. In our control cells, there was a significant decrease in cell survival rate following 50μm glutamate treatment that was comparable to the positive control (50μm glutamate =38.22±3.15%, n=8 coverslips; 250μm glutamate =31.23±3.78%, n=8 coverslips) (fig. 19a). Unexpectedly, neurons expressing only kif5c tail appeared to recover from 50μm glutamate insult (survival rate= 78.08 ±22.21%, n=8 coverslips) compared to the negative control (250μm glutamate) where survival rate was
down to 27.52 ±14.37%, n=8 coverslips (fig. 19b). Our results suggest that overexpression of kif5c tail alters the cellular response to excitotoxic challenge and may provide neuroprotection against such neurotoxicity.

**Figure 19. Global effect of overexpressed kif5c tail on cortical neuron viability following excitotoxicity**

Neurons were exposed to 50μm and 250μm glutamate in HBSS for 10 minutes at 37°C. Overexpression of exogenous kif5c tail appears neuroprotective to the effects of excitotoxicity. Neurons were less susceptible to the effects of glutamate neurotoxicity after a 24hr recovery period 78.08 ±22.21% survived (n=8 coverslips) compared to control where only 38.22±3.15% (n=8 coverslips) of the population survived, p-value<0.05
4. Discussion

In this study, I elucidate the effects of disrupted kif5c mediated mitochondrial movement on mitochondrial morphology, distribution and function. My results reveal that mitochondria morphology is altered to a shortened and rounded phenotype due to mitochondrial remodelling. I also demonstrate that mitochondrial functions are affected; ATP levels are relatively higher whereas ROS levels are lower. Finally, toxicity tests were performed on the primary cortical neurons and kif5c mitigated the effects of excitotoxicity.

4.1. Overexpression of Kif5c Tail Affects Mitochondrial Form/ Morphology

4.1.1. Effects on Primary Cortical Neurons

Overexpression of kif5c tail resulted in altered mitochondrial shape in primary cortical neurons. This is the first study to demonstrate that the expression of the motor protein kif5 affects mitochondrial morphology. Compared to the tubular mitochondria in control cells, neurons overexpressing kif5c tail where shortened in length and had a rounded shape (fig. 8a). The punctate shape of mitochondria was a direct consequence of the decreased mitochondrial length reported. It is important to note that no changes to mitochondrial diameter were observed. However, any possible changes to mitochondrial diameter were beyond the limits of our detection. It has been widely reported that mitochondrial morphology is maintained by the balance between fusion and fission (Huang et al., 2011; Rube and van der Bliek, 2004). Possible explanations for the change in mitochondrial shape are that either one or all of the proteins involved in fission was upregulated or that mitochondrial fusion was downregulated as a result of the overexpressed kif5c tail in neurons. Increased translocation of cytoplasmic drp-1 to mitochondria via increased calcium influx and overexpression of drp-1 in neurons have resulted in increased fragmentation and punctate mitochondria (Barsoum et al., 2006;
Han et al., 2008a). Similarly, depletion of mfn2 function using an shRNA knock down of mfn2 resulted in punctate mitochondria due to increased net fission in both neuronal and non-neuronal cells (Uo et al., 2009). Increased mitochondrial fission has been linked with bioenergetics failure and increased ROS production and suggests that mitochondrial morphology is important for cell health (Barsoum et al., 2006; Skulachev, 2001). In this scenario one would expect to see an increase in mitochondrial number in the processes, however we did not (fig. 8c). Furthermore, if there was an increase in fission or decrease in fusion, additional mitochondrial fragments would have to be transported to the cell body for degradation by mitophagy. While direct quantification of mitophagy was not performed in this study, there was no evidence of increased mitophagy in kif5 tail transfected cells. Consequently, it is unlikely that increased fission or decreased fusion is the genesis of the mitochondrial morphology changes reported here.

Alternatively, the change in shape may be due to mitochondrial remodelling. We reported that although mitochondrial occupancy within the processes decreased (fig. 8b), the number of mitochondria remained unaltered. If fission were upregulated, one would expect an increase in the total number of mitochondria in the neurons, but mitochondrial occupancy may increase or decrease depending on the degree of morphological change to mitochondrial length and the total number mitochondrial after fission. Interestingly, we found that the number of mitochondria remained unaffected. In conjunction with the reduction in mitochondrial length, occupancy and unchanged mitochondrial count per unit length of process, the more plausible explanation for the punctate shape is the occurrence of mitochondrial remodelling (rounding), not upregulated fission or downregulated fusion. In response to kif5c tail transfection, each mitochondrion retracted, resulting in a shortened mitochondrion that occupied less space in the neuronal process rather than undergoing fission to produce at least two daughter mitochondria. In addition to shortened mitochondria I noticed that mitochondria appeared brighter following kif5c tail transfection; this could also be a direct consequence of mitochondrial rounding. This observation is consistent with remodelling and argues against mitochondrial fission. Rounding of mitochondria resulted in condensation of the mitochondrial matrix, where the YFP is expressed, into a smaller volume resulting in the increased YFP intensity. The increased intensity also implies that both inner and outer mitochondrial membranes were intact as no leakage of the YFP signal from the matrix.
into the extracellular was detected. Alternatively, (and more plausibly) rounding of mitochondria would result in a greater volume of YFP signal being imaged in the same light pathway, thus resulting in increased YFP intensity. Previous studies have reported decreased mitochondrial length in parallel with an increase in mitochondrial signal intensity in primary cortical astrocytes treated with the calcium ionophore 4Br-A23187 (Tan et al., 2011). 4Br-A23187 was used to increase the levels of intracellular calcium in the cells. They showed that elevated calcium levels induced both mitochondrial remodelling and calcineurin dependent fission (Cribbs and Strack, 2007; Tan et al., 2011). Importantly, they were able to block mitochondrial fission when the astrocytes treated with the calcium ionophore was also treated with a calcineurin inhibitor (Tan et al., 2011). This observation supports the hypothesis that mitochondrial remodelling and fission are two distinct phenomena with different mechanisms. However, the mechanisms mediating mitochondrial remodelling remains unclear.

Our results also propose a novel role of the molecular motor kif5c in the regulation of mitochondrial morphology. Multiple motors of different polarities can simultaneously attach to a single cargo and microtubule during intracellular trafficking resulting in a tug-of-war which also promotes bidirectional movement (Chevalier-Larsen and Holzbaur, 2006; Gross et al., 2002). The direction of the organelle is determined by the dominant motor. The simultaneous binding of both active anterograde and retrograde molecular motor on mitochondria may cause the physical stretching of mitochondria by resulting in its typical elongated shape. When the kif5c tail is overexpressed in our cells, the mitochondrion is only attached to the cargo binding domain of the molecular motor and does not experience the tension associated with being anchored to microtubules by fully functional molecular motors (fig. 20). This lack of tensile force causes mitochondria to retract to a rounded shape. Interestingly, in isolated mitochondria preparations, they appear as rounded organelles (Claude and Fullam, 1945) supporting the hypothesis that mitochondria are stretched by molecular motors to facilitate the elongated shape. In addition, similar punctate mitochondria with reduced mitochondrial length were observed when the dynein/dynactin mediated mitochondrial movement was impaired in neurons and astrocytes by the overexpression of the CC-1 region of the p150glued subunit of dynactin (Lardizabal, 2010).
Figure 20  Schematic of proposed mechanism of kif5 mediating mitochondrial morphology through the process of mitochondrial remodelling.

We demonstrated that the magnitude of the effect of kif5c tail on mitochondrial morphology was affected by the relative levels of kif5c tail overexpressed by the neurons (fig. 9). Based on the tug of war model where multiple motor are simultaneously attached on mitochondria and the concept that the motors physically stretch mitochondria, we propose that the level of kif5c tail expressed progressively affects the mitochondrial shape. There is an inverse relationship between exogenous kif5c tail expression and mitochondrial length. In the presence of low kif5c tail expression, there is less impairment of functional endogenous kinesin-1 motors and mitochondria are maintained in their native, elongated shape similar to that seen in control neurons. Conversely,
when high levels of kif5c tail are overexpressed, there is severe impairment of more functional endogenous kinesin-1 motors resulting in a distinct change in mitochondrial morphology to a more rounded form.

In summary, we propose that mitochondrial morphology may be mediated by the kif5 molecular motor regulated through a mechanism that is still undefined. However, based on our findings we propose that the kif5 motor produces a tensile force, in concert with the dynein/dynactin molecular motor, which maintains the elongated mitochondria shape. The disruption of this tension results in the remodelling (rounding) of mitochondria. Since mitochondrial numbers are not affected, only mitochondrial length, it is clear that this mechanism is not related to the better known phenomenon of mitochondrial fission.

4.1.2. **Effect on Primary Cortical Astrocytes**

Accumulation of mitochondria in the perinuclear region was observed in kif5c tail overexpressing astrocytes compared to control astrocytes (fig. 5a-b). This perinuclear distribution of mitochondrial is consistent with impaired anterograde transport of mitochondria. In primary culture, astrocytes have one MTOC with the plus-end of microtubules extending towards the periphery (Ciesielski-Treska et al., 1982; Hesketh et al., 1984). Mitochondrial distribution is perturbed as a result of overexpression of kif5c tail such that after biogenesis, transport to the periphery mediated by kif5c is diminished and resulted in the accumulation of mitochondria in close proximity to the nucleus. Similarly perinuclear distribution has been observed in embryonic cells after kif5b disruption (Tanaka et al., 1998) and NIH3T3 cells transfected with an anti-sense KBP, a protein involved in kif1b mediated mitochondrial movement (Nangaku et al., 1994; Wozniak et al., 2005). Perinuclear distribution of mitochondria implies insufficient ATP distribution throughout the cell. This in turn may result in the bioenergetic starvation of the peripheral regions of the astrocytes. This may impair active peripheral processes such as cellular migration or even cellular health. We observed no obvious cell death in astrocytes following kif5c tail transfection; however we noted that some of the astrocytes expressing kif5c tail were smaller in cell size. It is possible that the perinuclear clustering of mitochondria resulted in the bioenergetic starvation of the peripheral regions causing contraction of the cells, although further experiments would be necessary to test this
hypothesis. Another explanation may be that only smaller sized astrocytes are capable of surviving the impediment to normal kif5c function. In parallel with shortened mitochondria and their perinuclear distribution, smaller astrocytes may have lesser bioenergetic demands and as such are better able to meet their metabolic needs to sustain cellular function.

Similar to the effects seen in primary cortical neurons, overexpression of kif5c tail altered mitochondrial morphology from an elongated to a shortened punctate organelle, and mitochondria appeared brighter compared to control (fig. 5c). As previously stated, it suggests a unique role of kif5c in maintaining and/or regulating mitochondrial morphology (see previous section for further details). The kif5 molecular motors exert force on mitochondria when anchored to microtubule that results in the elongated shape typical seen in neurons compared to the punctate shape in kif5c tail overexpressing neurons or in isolated mitochondria (Claude and Fullam, 1945).

4.2. Kif5c Tail Overexpression Impairs Mitochondrial Movement

To impair endogenous kif5 function, kif5c tail was overexpressed in primary cortical neurons and astrocytes. Overexpression of exogenous kinesin tail domain results in competitive inhibition of endogenous kif5 and/or autoinhibition. Competitive inhibition was achieved by kif5c tail competing with functional kif5 for the ability to bind mitochondria. When a mitochondrion gets bound to the kif5c tail construct, transport is impossible as the construct lacks the head region that binds to microtubule and promotes processivity by ATP hydrolysis. In addition, the absence of cargo on any endogenous kif5 induces conformational changes that results in folding of the protein resulting in autoinhibition (Coy et al., 1999; Kaan et al., 2011). Autoinhibition is the result of a folded conformation of the kinesin motor such that the tail region binds the head region of the motor and detaches the motor from the microtubule network and returns to the cytoplasmic pool (Coy et al., 1999; Friedman and Vale, 1999; Goldstein, 2001). This prevents the energetically wasteful process of microtubule based movement of kinesins without bound cargo. Exogenous kif5c tail impedes mitochondrial motility by binding to the head region of endogenous kinesin-1 which is already bound to the microtubule
network. This newly formed complex consisting of the kinesin head domain, microtubule and kinesin tail domain disrupts the ATPase function of the head region by slowing the detachment of the trailing leg from the microtubule (Coy et al., 1999). As stated previously, kif5c tail is capable of inhibiting all kif5 isoforms (kif5a, kif5b, kif5c) (Konishi and Setou, 2009; Miki et al., 2001).

4.2.1. Effects on Primary Cortical Neurons

Using an Excel macro we were able to report the bulk movement of mitochondria. Bulk movement is the measure of non-directional movement of mitochondria based on the changes in mitochondrial edge detection between successive frames. The bulk movement observed in control neurons is consistent with the numbers reported by Rintoul et al. in their study on the effect of mitochondrial uncoupler trifluorocarbonylcyanide phenylhydrazone (FCCP) on mitochondrial movement (Rintoul et al., 2003). Furthermore, the decrease in bulk movement associated with overexpression of kif5c tail (fig. 10c) suggests that disruption of kif5 mediated movement results in impaired anterograde movement which mitigates the delivery of newly synthesized mitochondrial components from the perinuclear region. Failure to distribute mitochondria to regions of energetic demand could potentially result in impaired cellular health due to insufficient ATP supply, or elevated intracellular calcium due to improper calcium buffering. Similar studies disrupting mitochondrial movement achieved by overexpressing a dominant negative form of the dynein/dynactin complex in cortical neurons also resulted in the reduced of bulk movement compared to the control cells (Lardizabal, 2010).

Although we observed a significant increase in the percentage of mitochondria in motion, neurons overexpressing kif5c tail still appeared to have mitochondria in motion within the typical range of observed neuronal mitochondrial in motion (table 1). Generally, the reported percentage of mitochondria in motion in neurons is between 20-33% of the total mitochondrial population, although up to 47% of mitochondria in motion were observed in axons compared to dendrites (Beltran-Parrazal et al., 2006; Ligon and Steward, 2000; Misgeld et al., 2007; Overly et al., 1996). Mitochondrial movement, distribution and morphology have been shown to vary based on the maturity of cortical neurons (Chang and Reynolds, 2006a). Immature neurons (DIV 5) had shorter
mitochondria, less mitochondrial occupancy and were faster compared to mature neurons (DIV14). Shorter mitochondria are also more frequently in motion (Misgeld et al., 2007). This collective phenotype of mitochondria displayed in immature neurons supports the increased trafficking required during neuronal development for processes such as synaptogenesis and axonal outgrowth (Chang and Reynolds, 2006a). Mitochondrial movement in mature neurons is relatively more stable and less dynamic. Since neurons are post-mitotic cells, the requirements for mitochondrial motility change from support for process extension, synaptogenesis etc. during maturation to the maintenance of the established architecture and synaptic connections in mature neurons (Chang and Reynolds, 2006a). Interestingly, our data were not consistent with some of these findings. Our kif5c tail overexpression neurons had shortened mitochondria as well as reduced movement. This suggests that the delivery of mitochondria to regions of bioenergetic demand may be delayed. Also, the shortened mitochondria may be less efficient at ATP generation and distribution. The scope of our study does not involve an assessment of effect of overexpressing kif5c tail on the shape of the cristae. However, it has been shown that cristae shape can affect the assembly of the electron transport chain complexes and in turn affect the efficiency of ATP production through oxidative phosphorylation (Cogliati et al., 2013). Interestingly, mitochondrial condensation resulted in the expansion of the cristae space (Hackenbr.Cr, 1968; Hackenbrock, 1966). Disrupted energy delivery may in turn affect active trafficking of mitochondria resulting in an endless loop of energy depletion (Duchen, 2004; Nicholls, 2005). Additionally, calcium buffering may be impaired as mitochondria may not be delivered to region of high calcium concentration leading to an accumulation of calcium in the cytoplasm. (Pivovarova and Andrews, 2010) This could result in the overactivation of numerous calcium dependent signalling pathways. There may also be a reduced capacity to service multiple synapses due to the decrease in surface area associated with the reduction in mitochondrial length. These characteristics could lead to cellular injury and ultimately cellular death.

In addition to impairments in bulk movement, kif5c tail overexpressing neurons exhibited a decline in the straightline velocity of mitochondria (fig. 10e). This may be due to diminished processivity of endogenous kinesin as a result of binding of the kif5c tail to the head region. The velocity typically generated by kinesin-1 during motion varies
between 0.4μm/sec to 0.8μm/sec (Beltran-Parrazal et al., 2006; Pilling et al., 2006; Vale et al., 1985; von Massow et al., 1989). We reported higher mitochondrial velocities in our study (kif5c tail=0.69±0.07μm/sec, control=1.06±0.14 μm/sec). Overexpression of kif5c tail increases the pool of kinesin, and previous studies have reported an increase in velocity due to a similar increase in the density of cytoplasmic pool of kinesin (Bohm et al., 2000). Within neurons fast anterograde axonal transport has a wider range varying between 1.8μm/sec and 5μm/ sec (Brady, 1991; Brady et al., 1990; Howard, 1996). This may account for the relatively high velocities generated in both kif5c tail expressing neurons and control cells. It is important to note that in our study, we did not distinguish between anterograde versus retrograde or axonal versus dendritic movement of mitochondria.

Impaired mitochondrial movement has been implicated in various neurodegenerative diseases (Hirokawa et al., 2010; Hirokawa and Takemura, 2004). Mitochondrial trafficking is important to support other mitochondrial dynamics such as fusion (and consequently mitochondrial quality control). Following damage or depolarization to mitochondria, fusion can restore normal function to damaged mitochondria by facilitating mixing and complementation of mitochondrial contents and DNA (Chen and Chan, 2005). Apposition of mitochondria is necessary for fusion; and this process is orchestrated by the action of molecular motors. Elimination or impairment of mitochondrial movement may prevent mitochondrial fusion events resulting in the accumulation of damaged mitochondria and/or mtDNA, increased ROS generation leading to oxidative stress and dysfunctional mitochondria. (Arduino et al., 2012; Cardoso et al., 2012; Twig et al., 2008; Youle and van der Bliek, 2012). Accumulation of dysfunctional mitochondria can further lead to compromised cellular health and eventually death.

4.2.2. Effects on Primary Cortical Astrocytes

In contrast to the effects in observed in primary cortical neurons, mitochondrial movement in primary cortical astrocytes was unaffected in astrocytes overexpressing kif5c tail compared to control cells. (fig. 5d) It is unclear why mitochondria bulk movement is unchanged when perinuclear distribution of mitochondria is observed after overexpression of kif5c tail. One possible explanation may be the orientation of
microtubules in astrocytes. Although microtubule arrangement from the MTOC is similar to neurons, polymerizing only on the plus-end, microtubule orientation in astrocytes has not been explicitly reported in the literature (Ciesielski-Treska et al., 1982; Goetschy et al., 1986; Hesketh et al., 1984). It is assumed that microtubule orientation in astrocytes is mixed similar to the arrangement observed in dendrites. This may mask a decline in bulk movement, as dynein/dynactin mediated mitochondrial movement is still active within the astrocytes.

Another explanation may be that kif5 is not an important motor for mitochondrial movement in astrocytes. Only one of the isoforms of kif5, kif5b, is expressed in astroglial cells although all 3 isoforms show redundancy in function (Kanai et al., 2000). Similarly, microtubule-based trafficking may not be important for intracellular trafficking of mitochondria in astrocytes; rather actin-based transport via myosin may be responsible. However, this is unlikely because actin is thought to provide the driving force for cellular migration and membrane extension through its formation of the lamellipodia and filopodia at the leading end of the astrocytes (Pollard and Borisy, 2003). Interestingly, microtubules are involved in regulating the dynamics of actin involved in astrocyte migration (Dunn et al., 1997).

4.3. Kif5c Disruption Affects Other Cargo

Kinesin-1, kif5, is important for intracellular trafficking. Although the kif5 molecular motor is responsible for most mitochondrial movement, it is also responsible trafficking of other cargo vesicles including amyloid precursor protein (APP), apolipoprotein E receptor 2 (APOER2), mRNA containing granules, lysosomes and tubulin oligomers (Hirokawa and Takemura, 2005; Kamal et al., 2000; Nakata and Hirokawa, 1995; Terada et al., 2000; Verhey et al., 2001). Cargo binding specificity to the kif5 motor is determined by various factors including the specific adaptor proteins involved or and direct interaction with the KLC or the KHC (Hirokawa et al., 2009; Hirokawa and Takemura, 2004; Setou et al., 2002). We evaluated whether the effect of overexpression of kif5c was specific to mitochondria or resulted in a global disruption of intracellular trafficking. Our results showed that the distribution pattern of α-tubulin and the autophagosome marker, LC3, were undisrupted (fig. 12 & fig. 14). These findings
confirm that 1) the effect of kif5c tail expression on mitochondrial movement is not due to an impaired or compromised cytoskeletal structure of the neurons as reflected by the stable organization of microtubules 2) the process of mitophagy is not disrupted as a result of disrupted kif5 movement. Mitophagy is important for the degradation of damaged component and the recycling of amino acids, therefore insufficient or excessive mitophagy is dangerous to cellular health and is implicated in aging and neurodegeneration (Mizushima et al., 2008). Autophagy (and mitophagy) is coordinated by a number of conserved autophagosome genes (atg) (Kabeya et al., 2000). Under physiological conditions, newly synthesized LC3 is in its cytoplasmic form (LC3-I) (Ladoire et al., 2012). However, when mitophagy is stimulated, formation of the autophagosome membrane is promoted and LC3 is recruited to the membrane resulting in an LC3 decorated autophagic puncta (LC3-II) (Kabeya et al., 2004). Our LC3 is capable of detecting both forms of the LC3 protein. Our results show cytoplasmic dispersion of LC3, indicating LC3 is still in its soluble form in both groups. Although we disrupted kif5 mediated mitochondrial movement, this treatment does not appear to promote mitochondrial damage as evidenced by the lack of LC3 puncta in treated cells. This suggests that mitophagy is not upregulated by kif5c tail overexpression. However, the distribution of lysosomes appears to be affected as evidenced by the effects of kif5c tail overexpression on LAMP1 (fig. 13). One crucial function of the lysosomal associated membrane protein 1 (LAMP1) is to protect the cytoplasmic contents from unwanted degradation by the hydrolases contained within lysosomes. Damaged proteins are targeted to lysosomes for hydrolytic degradation (Braulke and Bonifacino, 2009). Under physiological conditions, lysosomes are localized to the perinuclear regions of the cell (Huynh et al., 2007). Lysosomal distribution within neurons changes during the development; lysosome become less concentrated within the dendrites and axon and more accumulated within the perinuclear region as the hippocampal neurons mature (Roberts and Gorenstein, 1987). Our control cells shows an accumulation of lysosomal marker within the perinuclear region as well as a scattering of the LAMP1 markers within neuronal processes similar to pattern reports in other studies (Lee et al., 2011). We show that disruption of lysosomal puncta in kif5c tail transfected neurons was less compared to control cells. This suggests that the delivery system of lysosomes was perturbed by the overexpression of kif5c tail. This may be detrimental to cellular health as damaged cellular contents may accumulate due to the dysfunction of lysosomal
pathways. Disruption of lysosomal trafficking by kif5c tail overexpression is likely to have negative consequences on these cells but our present data do not permit further speculation. It is important to note that lysosomes are capable of bidirectional movement which is mediated by both kinesin and dynein molecular motors (Heuser, 1989). This feature of bidirectionality may permit some compensatory mechanism during disrupted movement in one direction similar to tests in our study where we disrupted kif5 mediated movement of lysosome. Alternatively, negative consequences may develop over a longer time frame (i.e. >24 hours) than was conducted during our study.

4.4. Kif5c Disruption Affects Mitochondrial Function

4.4.1. ROS Generation

Characterization of Ro-GFP in Primary Cortical Neurons

Maintenance of the balance between ROS generation and removal are crucial for the health of the cell. In our study we used a novel ROS sensitive probe, ro-GFP, to evaluate the redox state of primary cortical neurons to provide insight on cellular function. The change in the spectral properties of the ro-GFP probe is used to determine the relative ROS levels in the cell; in an oxidized state, peak excitation is in 380nm wavelength compared to 492nm when in a more reduced state. We used a version of ro-GFP that is expressed evenly throughout the cytoplasm of the cell. To our knowledge, only one other study has shown the use of ro-GFP in neurons to monitor the range of sensitivity of the ro-GFP probe (Funke et al., 2011). Using primary cortical neurons, we were able to characterize the functional range of ro-GFP in response to fluctuating ROS dynamics when an exogenous reductant or oxidant was applied to the cells (fig. 17).

100μm H₂O₂ was used as the oxidizing agent in our experimental paradigm. There was a spike in the 380nm/492nm ratio as a result of perfusing neurons with 100μm H₂O₂ indicative of intracellular oxidation. Although we used H₂O₂, ro-GFP is capable of sensing other types of ROS including superoxide, the primary ROS generated by the mitochondria (Funke et al., 2011). Superoxide is generally short-lived and gets converted to H₂O₂ spontaneously or through catalysis by superoxide dismutase. 1mM DTT was perfused onto the cells to stimulate a reduced intracellular
environment. There was gradual drop in 380nm/492nm a result of the reduction caused by DTT. DTT affects proteins by breaking the disulphide bonds in cysteine residues and replacing them with thiol groups through the thiol-disulphide exchange reaction (Kim et al., 1985).

Collectively, the data establishes the range of sensitivity of ro-GFP during intracellular fluctuation of redox states in primary cortical neurons. We also demonstrate that ro-GFP is reversible unlike alternative ROS sensitive tools like the fluorescent dye 5-carboxy-2’,7’-dichlorofluorescein diacetate (DCFDA) which is irreversible after oxidation (LeBel et al., 1992; Zhu et al., 1994). Addition of 1mM DTT was able to reverse the oxidation by 100μm H₂O₂ as reflected by the change in the 380nm/492nm ratio. Additionally when HBSS was used to washout the 1mM DTT, 380nm/492nm increased but did not recover fully to baseline levels observed. This may be due to the use of DTT specifically. DTT is considered a particularly strong reducing agent. Although the actions of DTT are reversible, once oxidized via the thio-sulphide exchange reaction, it forms a six-membered ring with an internal disulphide bonds (Hansen and Winther, 2009). The oxidized form is more stable than its reduced form as the reduced form can also undergo oxidation by atmospheric oxygen. This property makes it harder for DTT to revert the oxidized form back to its reduced form. In combination with the high concentration of DTT used during perfusion, this may account for the unsuccessful full recovery of ROS levels to baseline line after the HBSS washout. Alternatively, the rinse out time for DTT during the perfusion may have been insufficient to completely wash out the chemical. Nevertheless, our results demonstrate the utility of ro-GFP as a tool to measure redox reactions in live cultured cortical neurons as they occur in real time.

**Effect of Overexpressing Kif5c Tail on ROS Levels in Primary Cortical Neurons**

A by-product of oxidative phosphorylation in mitochondria is ROS. Although it was previously thought to be only detrimental, it is now acknowledged that ROS is involved in various cell signalling pathways that promote cellular health including cell cycle, proliferation, apoptosis, oxygen sensing, transcription factors, kinases and phosphatases (Bolisetty and Jaimes, 2013). Despite their importance in many normal cellular functions, ROS is associated with the pathophysiology of a plethora of neurodegenerative diseases including Alzheimer’s and Parkinson’s Disease (Manton et
The balance of ROS levels within cells and control by detoxifying proteins and enzymes is highly regulated (Andreyev et al., 2005). In mitochondria, superoxide dismutase is the first line of defence against ROS generated during cellular respiration. Our results showed a decrease in ROS levels in neurons overexpressing kif5c tail compared to control (fig. 18). Although there is no known link between ROS generation, mitochondrial trafficking and mitochondrial morphology, we speculate the punctate shaped mitochondria generate less ROS as a result of their reduced volume, compared to elongated mitochondria. However, we would require transmission electron microscopy to positively identify the effects of punctate shape on the inner mitochondrial membrane which contains the component complexes of the electron transport chain and further biochemical measures to assess the levels activity of the electron transport chain.

Astrocytes are the most abundant glial cell type in the central nervous system and they have a higher ROS detoxification capacity compared to neurons (Sheng et al., 2013). Our primary tissue culture was a mixed population containing both neurons and astrocytes. The use of the Neurobasal/B-27 media reduces the glial content to approximately 0.5% of the total neuron population (Brewer et al., 1993). Astrocytes provide support for neurons by the uptake of glutamate in the synaptic cleft protecting neurons from the effects of potential excitotoxicity, and recycle glutamate back to the neuron (Makar et al., 1994b; Yi and Hazell, 2006). Astrocytes also protect neurons against oxidative stress caused by ROS. Previous studies have shown that neurons cultured in the presence of astrocytes have increased resistance to toxic oxidative stressors (Gegg et al., 2003; Langeveld et al., 1995; Tanaka et al., 1999). Astrocytes have higher levels of ROS detoxifying enzymes and antioxidants including glutathione (GSH) compared to neurons (Makar et al., 1994a; Wilson, 1997). Based on these previous reports we speculate that the effects on ROS we observed due to the overexpression of kif5c tail in the neurons may be an underestimation of the true effects of ROS generation as our culture contains astrocytes capable of detoxifying ROS. However, we would require further testing to quantify the levels of GSH or other ROS detoxifying enzymes in our culture to confirm if there is an upregulation of ROS detoxification in the neurons overexpressing the kif5c tail.
4.4.2. ATP Production

Characterization of ATeam in Primary Cortical Neurons

Most intracellular ATP is generated from glycolysis which occurs in the cytoplasm and oxidative phosphorylation in mitochondria. Due to the high metabolic demands of neurons, they are almost exclusively reliant on the oxidative phosphorylation system of mitochondria to meet their energy needs. Fixed and live cells studies have shown that mitochondria are typically localized to sites of high metabolic demand such as nodes of Ranvier, growth cones, synapses and myelination/demyelination interfaces (Chang and Reynolds, 2006b). This is because of the high efficiency of mitochondrial ATP generation via oxidative phosphorylation in the electron transport chain in comparison to glycolysis. We used a FRET-based ATP probe, ATeam, to monitor the relative levels of ATP in neurons in real time. When ATP is bound to the probe, a conformational change occurs which permits the efficient transfer of resonant energy from the CFP fluorochrome to the YFP fluorochrome (Imamura et al., 2009). Therefore in the presence of ATP there is a higher 535nm/465nm emission ratio. We characterized the range of sensitivity of ATeam in neurons (fig. 15). We established a baseline ratio in cells perfused with HBSS alone which was decreased by the addition of potassium cyanide (KCN). KCN is an inhibitor of cellular respiration which acts on complex IV of the electron transport by preventing oxygen consumption. These results also confirm the importance of oxidative phosphorylation as the primary source of ATP generation in neurons. There was more than a 50% reduction in the level of cytoplasmic ATP present in the cells within 10mins of treatment with KCN.

Effect of Overexpressing Kif5c Tail on ATP Generation in Primary Cortical Neurons

Microtubule-based intracellular trafficking is a ATP-dependent mechanism (Hirokawa and Takemura, 2005). Because neurons are highly excitable cells that whose neuronal processes extend large distances, they required efficient ATP supply. Their energetic demand is met by the ATP generated in mitochondria which is trafficked to region of high metabolic demand. Because of their high bioenergetic needs neurons are highly susceptible to impaired mitochondrial trafficking (De Vos et al., 2008). Slow ATP diffusion from the site of generation, in the absence of mitochondria movement, is not
sufficient to sustain neuronal function (Goldstein et al., 2008). Unexpectedly, when we overexpressed kif5c tail in neurons, we observed a small but significant increase in the relative ATP levels compared to control neurons (fig. 16). This observation is inconsistent with other studies that suggest that the elongated morphology of mitochondrial may confer some bioenergetic advantage with regards to ATP generation and delivery (Skulachev, 2001). Precisely how impaired mitochondrial transport leads to elevated intracellular ATP levels is unclear. One potential explanation involves the observation that molecular motors undergo ATP hydrolysis during processivity thereby depleting cellular ATP. It is possible that the disruption of functional endogenous kif5 increases the ATP reserve pool since active trafficking is reduced and less ATP is required. However, the increase in ATP levels in kif5c tail overexpressing neurons is inconsistent with the decreased levels of ROS that we observed.

4.5. Kif5c Attenuates the Effects of Excitotoxicity on Primary Cortical Neurons Survival

Our data suggests that overexpression of kif5c tail provides neuroprotection against excitotoxicity (fig. 19). Excitotoxicity is the pathological process where excessive stimulation of the N-methyl-D-aspartate receptor (NMDA-R) by the elevated extracellular level of the neurotransmitter glutamate results in a massive increase in intracellular calcium (Olney, 1986; Olney et al., 1986a; Olney et al., 1986b) The elevated intracellular calcium levels results in the massive potential-driven calcium influx into the mitochondria through the calcium uniporter; this eventually dissipates the potential force driving ATP generation, increases ROS production and leads to the release of pro-apoptotic proteins and the activation of caspases that result in apoptosis and cell death (Dugan et al., 1995; Green and Kroemer, 2004; Reynolds and Hastings, 1995).

Other studies have shown that mitochondrial movement is inhibited by FCCP, oligomycin, zinc, nitric oxide and excess glutamate (Malaiyandi et al., 2005; Rintoul et al., 2006; Rintoul et al., 2003). Initially we demonstrated that kif5c disruption also resulted in reduced movement, as well as shorted mitochondria and altered mitochondrial distribution (see previous sections for details). Unexpectedly, when we challenged the neurons with a glutamate stressor, a higher percentage of neurons
overexpressing kif5c survived compared to control cells (fig. 19). Glutamate-induced neuron death requires mitochondrial calcium uptake (Stout et al., 1998). When mitochondrial calcium uptake was prevented using mitochondrial inhibitors (FCCP) that promoted mitochondrial depolarization, neuronal survival was improved following glutamate toxicity treatments despite the elevated cytoplasmic calcium concentration (Stout et al., 1998). It is possible that the redistribution of mitochondria caused by kif5c tail affects the ability of mitochondrial to buffer intracellular calcium thus protecting the cells from death by excessive glutamate. Characterization of the mitochondrial calcium uniporter (MCU) showed it has high selectivity towards calcium ions relative to other ions tested (Kirichok et al., 2004). MCU also has low affinity to cytoplasmic calcium and thus requires high concentration of calcium to permit loading (Carafoli, 2012; Raffaello et al., 2012; Werth and Thayer, 1994). Therefore, as a result of altered distribution and shortened of mitochondria by kif5c tail overexpression, mitochondria may be positioned further away from high calcium microdomains within the neuron and are consequently not as able to take up calcium. This redistribution results in a form of neuroprotection as the effects of excitotoxicity due to massive uptake of calcium are reduced and neurons are able to survive the toxic insult, at least over the 24 hour period post stimulus which our studies examined.

In addition to the disruption of mitochondrial movement by excess glutamate, studies have shown shortening of mitochondria caused by both fission and remodelling (although distinction between both phenomena were not made) in part due to calcium-dependent pathways after the cells were glutamate treatment (Han et al., 2008b; Ligon and Steward, 2000; Rintoul et al., 2003; Tan et al., 2011). Other studies have shown that elevation of intracellular calcium via 4Br-23187 in neurons and astrocytes resulted in a change in mitochondrial morphology to punctate shapes (Dubinsky and Levi, 1998; Kristal and Dubinsky, 1997). Another possible explanation regarding the neuroprotection by kif5c tail overexpression is that our glutamate treatment resulted in additional shortening of mitochondrial length, than with just kif5c tail alone, thereby further reducing the surface area of mitochondria available for calcium uptake into the mitochondria.

Mitochondrial movement is regulated by the levels of intracellular calcium (Rintoul et al., 2003; Yi et al., 2004). Yi et al. showed inhibition of mitochondrial movement in response to elevated intracellular calcium, which was released from the
endoplasmic reticulum in myoblasts (Yi et al., 2004). This arrest assists in the recruitment and localization of mitochondria to active calcium release sites, thus enhancing mitochondrial calcium buffering functions. The uptake of calcium by mitochondria may also serve as a feedback signal stimulating localized generation of ATP, which is used by the ER Ca\(^{2+}\)-ATPase for calcium reuptake (Landolfi et al., 1998).

Mitochondrial remodelling and the reduced mitochondrial trafficking may act synergistically to provide neuroprotection to the cells. The combination of lower levels of ROS, higher ATP levels and the altered distribution of mitochondria (which prevented the import of calcium into mitochondria following glutamate treatment), produced by overexpression of kif5c tail may act in concert to produce the neuroprotective qualities observed. It is noteworthy that these are relatively acute effects of kif5c disruption compared to the chronic disruption of mitochondrial trafficking (as may occur in slow onset neurodegenerative disease) which may ultimately be harmful to neurons.
5. Conclusion

Mitochondrial dynamics is a multifactorial process, the dysfunction of which has emerged as a key contributor to the pathophysiological processes underlying various neurodegenerative diseases (Sheng and Cai, 2012). It is still unknown whether mitochondrial dysfunction is the result of the disease or the cause. However, a clear understanding of how mitochondrial dynamics processes influence one another would bridge the gap in knowledge, lead to a better understanding of disease states, and eventually generate potential therapies. In essence, the unanswered question is: does mitochondrial form affect function or vice versa? Therefore, in an attempt to elucidate the impact mitochondrial movement has on mitochondrial function and susceptibility to exogenous stressors, we perturbed kif5 mediated mitochondrial movement. In our study we provide preliminary insight to demonstrate the effects of disrupted mitochondrial movement on mitochondrial form and function in real time.

Using a dominant negative form of kinesin-1, kif5c tail, we were able to impede mitochondrial movement mediated by all the kif5 motor isoforms. Overexpression of kif5c tail resulted in the shortening of mitochondria through a fission-independent mechanism. We are the first group to propose that the kinesin molecular motor regulates mitochondria morphology through a mechanism involving mitochondrial remodelling. Here kinesin-1 anchored to the microtubule network exerts a tensile force when it is concomitantly tethered to mitochondria. This results in the elongated morphology typical of mitochondria. Conversely, when endogenous kinesin-1 function is disrupted using kif5c tail, the motor loses the tension generated from being anchored to the microtubule network thus resulting in mitochondria reverting to a more spherical shape. Importantly we discovered that there was an inverse correlation between mitochondrial length and the expression levels of kif5c tail. This suggests that given a sufficient amount of time, kif5c tail overexpression could result in a more severe mitochondrial phenotype. This progressive deterioration would be similar to observations seen in slow-onset neurodegenerative diseases (Barsoum et al., 2006; Bossy-Wetzel et al., 2008; Knott and
Bossy-Wetzel, 2008; Knott et al., 2008). Altered mitochondrial morphology is observed in the pathology of neurodegeneration diseases. However, it is unclear whether they are the cause of the disease or the downstream consequence of the diseased state. We provide data that suggests mitochondrial movement directly affects the morphology of mitochondrial and this may be a potential target for treatment in diseases where mitochondrial form is altered.

A potential future direction of this study would be to determine the levels of the common mitochondrial morphology proteins (mfn1/2, OPA1, drp1 and fis1) to evaluate whether their relative protein expression levels are consistent with the shortened mitochondrial morphology. Rescue assays could be performed to determine if the change in mitochondrial shape can be transformed back to one that is elongated by the overexpression of endogenous kinesin-1 or dominant-negative fission proteins. Also, one could evaluate any potential role these proteins play to facilitate the motor-dependent remodelling we observed in our study. Finally, a study to evaluate the mechanism of mitochondrial remodelling is needed. Until recently mitochondrial remodelling has been unappreciated as another mitochondrial dynamic process. Further studies on this phenomenon can help explain if this process is neuroprotective or the consequence of dysfunction of mitochondrial environment. Alternatively, remodelling may represent a compensatory mechanism to mitochondrial pathology and thus represent a potential diagnostic tool for the early detection of neurodegenerative diseases.

Although we were unable to assess the impact of remodelling on the cristae, other studies have shown that cristae remodelling is affected by the mitochondrial morphology (Cogliati et al., 2013). This suggests that mitochondrial remodelling could affect mitochondrial function. However, the future direction of this study was benefit from an examination of the effect on cristae morphology, using electron microscopy, as a result of kif5c tail overexpression in neurons. We used novel fluorescent probes to determine the effect of disrupted kif5c movement on mitochondrial ROS and ATP generation: ro-GFP and ATeam respectively. The use of both ro-GFP and ATeam within the field has not been extensively characterized in neurons to determine the possible levels of detection by each probe. In this study we characterized of both probes in a neuronal model system. We found that disruption of kinesin-1 mediated movement
resulted in decreased ROS levels and increased ATP levels. A clear understanding of the link between mitochondrial movement and ROS and ATP generation should be the topic of future studies. In particular, longer time course of exposure to kif5c tail would elucidate whether the ROS/ATP changes reported are transient or progressive.

We also discovered that overexpression of kif5c tail conferred neuroprotection to neurons challenged with an excitotoxic stressor. These results are consistent with previous reports showing mitochondrial uptake of calcium as the essential step required for death during excitotoxic stress. The distribution pattern of mitochondria in kif5c tail transfected cells was altered and may have resulted in the absence of mitochondria in microdomains containing higher concentrations of calcium. Subsequent future experiments could examine localised levels of cytoplasmic calcium and mitochondrial calcium uptake after kif5c tail transfections to further investigate these phenomena. Additionally, both kinesin and the dynein/dynactin functions could be impeded and all mitochondrial parameters analysed by our study could be conducted. If our hypothesis is correct that kif5c disruption affects mitochondrial form, function and cellular susceptibility to neurotoxins, then we should observe a bigger change in mitochondrial morphology and subsequently greater effects on physiological measures when both motors are disrupted.

In conclusion, we believe that in the question of what comes first, form or function? Mitochondrial form appears to be the mediator of mitochondrial function and in turn cellular health. Mitochondrial morphology regulation could prove to be a potential therapeutic target in the pathology of neurodegenerative diseases.
References


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