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Identifying regulators of synaptic stability during normal healthy ageing

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Doctor of Philosophy

College of Medicine and Veterinary Medicine
University of Edinburgh
2017
Declaration

I declare that this thesis is an original report of my research, has been written by me and has not been submitted for any previous degree. The experimental work is entirely my own work and any collaborative contributions have been clearly indicated in the text. Due references have been provided on all supporting literatures and resources.

Laura C. Graham
September 2017
Abstract

The loss and dysfunction of selected populations of synapses is characteristic of mammalian brain ageing and alterations in these receptive compartments are considered to underpin age-related cognitive decline. Discrete neuroanatomical regions of the cortical architecture harbour disparate populations of synapses that demonstrate significant heterogeneity with regards to advancing age. Of particular interest is the hippocampus, which is selectively vulnerable during ageing. The hippocampal synaptic architecture exhibits subtle structural and biophysical alterations, which are considered to promote the manifestation of cognitive symptoms in aged patients. This notion of “selective synaptic vulnerability” has been the focal point of a multitude of morphological studies investigating age-related cognitive decline, which have often provided tentative conclusions as to how this phenomenon may be regulated. The molecular correlates bolstering the reported age-dependent morphological and functional shift remain elusive and studies are only now beginning to unravel how discrete organelles, proteins and signalling cascades may hierarchically or synergistically attenuate synaptic function. Until there is considerable comprehension of how functional mediators drive the biochemical substrates regulating age-related cognitive decline, there are limited strategic avenues for the development of efficacious therapeutic interventions that promote successful ageing.

To address the phenomenon of selective synaptic vulnerability, we have utilised an unbiased combinatorial approach, including quantitative proteomic analyses coupled with in vivo candidate assessments in lower order animals (Drosophila), to temporally profile regional synapse and synaptic mitochondrial biochemistry during normal healthy ageing. We begin by demonstrating that cortical mitochondria located at the synaptic terminal are morphologically distinct from non-synaptic mitochondria in adult rodents and human patients. Biochemical isolation and purification of discrete mitochondrial subpopulations from control adult rat forebrain enabled generation of synaptic and non-synaptic mitochondrial molecular fingerprints using quantitative proteomics, which revealed that expression of the mitochondrial proteome is highly dependent on subcellular localisation. We
subsequently demonstrate that the molecular differences observed between mitochondrial sub-populations are capable of selectively influencing synaptic morphology in-vivo. Next, we sought to examine how the synaptic mitochondrial proteome was dynamically and temporally regulated throughout ageing to determine whether protein expression changes within the mitochondrial milieu are actively regulating the age-dependent vulnerability of the synaptic compartment. Proteomic profiling of wild-type mouse cortical synaptic and non-synaptic mitochondria across the lifespan revealed significant age-dependent heterogeneity between mitochondrial subpopulations, with aged organelles exhibiting unique protein expression profiles. Recapitulation of aged synaptic mitochondrial protein expression at the Drosophila neuromuscular junction has the propensity to perturb the synaptic architecture, demonstrating that temporal regulation of the mitochondrial proteome may directly modulate the stability of the synapse in-vivo.

Although we had comprehensively characterised the temporal regulation of rodent cortical mitochondrial subpopulations, providing a number of novel candidates that may be mediating synaptic vulnerability during ageing, we sought to establish whether similar alterations were occurring in the primate brain. Using synaptic isolates from neuroanatomically distinct age-resistant (occipital cortex) and age-vulnerable (hippocampus) regions, we demonstrate that synaptic ageing is brain-region dependent and that discrete populations of synapses significantly differ at a biochemical level in the healthy human and non-human primate brain. Recapitulation of aged hippocampal protein expression with genetic manipulation in-vivo revealed numerous novel candidates that have the propensity to significantly modulate multiple morphological parameters at the synapse. Furthermore, we demonstrate that several of these candidates sit downstream of TGFβ1 and activation of the TGFβ1 signalling cascade in hippocampal synaptic populations drives the aberrant expression of selected candidates during ageing. Finally, we show that selective pharmacological inhibition of this pathway rescues synaptic phenotypes in multiple candidate lines. The data affirmed that activation of the TGFβ1 transduction pathway modulates synaptic stability and thus may contribute to the selective vulnerability of hippocampal synapses during ageing.
Lay Summary

Ageing is a natural and inevitable part of life, and our society is currently shifting towards an aged population. While ageing affects all cells in the body, its advancement can vary significantly between individuals and even differently within organs. Although many of the cellular changes that occur throughout the lifespan are considered normal, they also represent a major risk factor for disease. It is well established that aged individuals are more vulnerable to the development of disease but basic questions remain unanswered. For example, it is unclear what triggers the development of disease and how age may change our vulnerability to these processes. There is therefore a vital need to understand what distinguishes ‘normal healthy’ from ‘pathological’ ageing.

The brain is organized into many different regions, containing billions of highly interconnected nerve cells, called neurons. As we age, the brain loses some of the connections between cells, or synapses, which compromises neuronal function. Importantly, the loss or alteration of synapses occurs more frequently in certain brain regions as we age. For example, in aged individuals there appear to be large numbers of changes in synapses that populate a brain region called the hippocampus. This area is a major site of memory storage and synaptic alterations in the hippocampus often underlie age-related memory changes. However, there is little understanding as to why particular populations of synapses are vulnerable to age-related changes.

To address the question of synaptic vulnerability, we characterised the molecular changes occurring in synapses during normal healthy ageing. The study made use of both rodent brain tissue and archived, frozen non-human primate brain samples, from young adult, middle-aged and old healthy control animals. Non-human primate tissues were used due to the close genetic relationship to humans, which might provide results more relevant to human ageing. By isolating synapses from specific brain regions we were able to track particular molecular trends that may be associated with normal healthy aging or increased vulnerability to disease. With the
use of computer tools, we were able to identify candidate protein molecules that may be contributing to synaptic vulnerability during ageing.

In order to actively test the role of candidate protein molecules that change with age and affect synaptic vulnerability, we took advantage of the fruit fly model, as the basic structure of synapses is present in the nervous system of all animals. By using fruit flies as a convenient experimental tool, we could quickly establish whether changing the amount of specific proteins we identified from our rodent and non-human primate experiments can directly influence synapse form or function. By using this variety of techniques, our study has identified several new molecules that may affect the vulnerability of synapses during aging.

These findings provide new insights into how synapses age and how changes in the amount of specific proteins can influence the vulnerability of the synapse. This may be important for the early detection of degenerative disease and open up new avenues for therapeutic targets against degeneration.
Acknowledgements

First and foremost I would like to thank the members of the Wishart lab - Tom, Sam and Maica, for their unwavering support throughout the duration of this project.

I am extremely grateful to my supervisor Dr. Tom Wishart for providing such a supportive and stimulating environment to work in over the past 4 years. I thank him for entrusting me with ambitious projects and allowing me the scientific freedom to develop and direct my PhD. Without his continued guidance and encouragement, none of this would have been possible. I am also hugely thankful to Maica Llavero for being the most supportive and ambitious lab partner over the past 4 years. From starting our PhD studies together to building our very own fly lab, it has been an absolute joy to work with her everyday. I cannot wait to see what discoveries our postdoctoral positions bring! I would also like to thank Sam Eaton for all of the technical and non-technical support throughout the duration of my PhD. I am extremely grateful for all of the times she provided advice, encouragement or simply a shoulder to cry on.

I would also like to thank the numerous collaborators involved in generating the data presented in this thesis. I am extremely grateful to Dr. Giusy Pennetta for hosting me in her Drosophila based laboratory for an extended period during my second year. The skills I obtained here have contributed significantly to the quality of the project and, for that, I cannot thank her enough. I would also like to thank Dr. Henryk Urbanski and Dr. Steve Kohama for hosting me in their laboratory at Oregon National Primate Research Center and for generously donating non-human primate tissues for my regional synaptic ageing study. Additionally, I thoroughly appreciate the help provided by Douglas Lamont, Amy Tavendale, Samantha Kosto, Abdel Atrih and Dr. Michael Naldrett with mass spectrometry and proteomics. I am also grateful to Dr. Paul Skehel and Prof. Tom Gillingwater for helpful scientific discussions regarding numerous projects including those out with this thesis.
A huge thank you to the BBSRC EastBio Doctoral Training Project for funding my studies and affording me the opportunity to obtain my PhD.

The completion of this project would not have been possible without the unconditional support of my friends and family. A special thank you to my parents who have provided me with continuous encouragement, motivation and determination to pursue my scientific career. I cannot thank them enough for fostering my ambition and drive. Finally, I am so grateful to my husband Callum for providing continued comfort and support throughout the project. His company and thoughtful insights during the most challenging moments has allowed me to accomplish my goals.
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<th>Description</th>
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<tbody>
<tr>
<td>2D-DIGE</td>
<td>Two-dimensional difference gel electrophoresis</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AHP</td>
<td>After hyperpolarizing potential</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
</tr>
<tr>
<td>BDSC</td>
<td>Bloomington <em>Drosophila</em> stock center</td>
</tr>
<tr>
<td>BRP</td>
<td>Bruchpilot</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Calcium calmodulin-dependent kinase 2</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DAVID</td>
<td>Database for annotation, visualisation and integrated discovery</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DIOPT</td>
<td>DRSC integrative ortholog prediction tool</td>
</tr>
<tr>
<td>DLG</td>
<td>Discs large 1</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMS</td>
<td>Delayed non-matching to sample</td>
</tr>
<tr>
<td>EC</td>
<td>Entorhinal cortex</td>
</tr>
<tr>
<td>ELAV</td>
<td>Embryonic lethal abnormal vision</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
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<tr>
<td>GOF</td>
<td>Gain of function</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity Pathway Analysis</td>
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<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>LOF</td>
<td>Loss of function</td>
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<tr>
<td>LTD</td>
<td>Long term depression</td>
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<tr>
<td>LTP</td>
<td>Long term potentiation</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MSB</td>
<td>Multi-synaptic bouton</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>nDNA</td>
<td>Nuclear DNA</td>
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<tr>
<td>NHP</td>
<td>Non-human primate</td>
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<tr>
<td>NMAD</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
</tr>
<tr>
<td>NSB</td>
<td>Non-synaptic bouton</td>
</tr>
<tr>
<td>NT</td>
<td>Neurotransmitter</td>
</tr>
<tr>
<td>PANTHER</td>
<td>Protein analysis through evolutionary relationships</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBT</td>
<td>Phosphate buffered saline + Triton X-100</td>
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<tr>
<td>PCA</td>
<td>Principal components analysis</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
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<td>PP1</td>
<td>Protein phosphatase 1</td>
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<tr>
<td>PSD</td>
<td>Postsynaptic density</td>
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<td>QFWB</td>
<td>Quantitative fluorescent western blotting</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SSB</td>
<td>Single synaptic bouton</td>
</tr>
<tr>
<td>SSR</td>
<td>Subsynaptic reticulum</td>
</tr>
<tr>
<td>SV2A</td>
<td>Synaptic vesicle glycoprotein 2A</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TRiP</td>
<td>Transgenic RNAi project</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream activating sequence</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin-proteasome system</td>
</tr>
<tr>
<td>VDRC</td>
<td>Vienna Drosophila resource centre</td>
</tr>
<tr>
<td>Wld</td>
<td>Slow wallerian degeneration</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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</table>
Chapter 1. Introduction

1.1 Ageing: a global epidemic?

Population ageing has become a twenty-first century global phenomenon. There has been a dramatic demographic shift worldwide and recent reports have highlighted that the number of individuals aged 80 years or over has increased by 76% since the year 2000 (1). Although this increase in lifespan may be deemed somewhat triumphant, reflecting superior healthcare strategies, technological advancements and innovative scientific discoveries, the prevalence of dementia and age-related cognitive decline is irrepressibly escalating. The significant surge in individuals afflicted with alterations in cognitive capacity poses an enormous societal, emotional and economic burden, accruing care costs upwards of $8 billion per year in the UK alone (2). This is projected to increase by up to 300% in the upcoming decade indicating an impending public health crisis (1, 2). With substantial healthcare challenges imminently approaching there is an urgent requirement for efficacious treatments that promote successful ageing.

Advancing age is the single largest risk factor for the development and diagnosis of a neurodegenerative disease. How the dichotomous pathways regulating normative ageing and pathogenic ageing intersect remains elusive, however investigating spatiotemporal changes in the “normal” central nervous system (CNS) milieu may reveal molecular pathogenic precursors that have the propensity to significantly perturb neuronal structure and function. As humans age, the brain exhibits subtle cellular and molecular alterations that progressively impair neuronal receptivity to novel stimuli and enhance susceptibility to pathogenicity. These physiological modifications manifest as a selective decline in multiple cognitive faculties, particularly those regulated by the hippocampus e.g. executive function, short-term memory capacity and retention of novel information (3-5), causing patients significant concern. Until recently, age-related cognitive decline was purportedly an inevitable component of normative ageing, however emerging evidence suggests that
the trajectory of ageing displays significant malleability (6-8). The advent of genomic, transcriptomic and proteomic technologies has enabled the identification of discrete signalling cascades, effector molecules and genetic transcripts that alter during the lifespan suggesting cognitive preservation via the manipulation of molecular mediators may be a tangible possibility. Although an auspicious notion, investigations have detected exceptionally few functional mediators capable of modulating or ameliorating age-related cognitive decline in vivo (7). Identification of such functional mediators is a critical junction that must be resolutely attained in order to provide mechanistic insights into the disrupted molecular pathways that may be constitutively promoting age-dependent cognitive decline and/or pathogenic neurodegenerative cascades. Until there is considerable comprehension of how functional mediators hierarchically or synergistically drive the biochemical substrates regulating age-related cognitive decline, there are limited strategic avenues for the development of efficacious therapeutic interventions that promote successful ageing.
1.2 The synapse

1.2.1 Synaptic morphology

The brain is a highly complex and sophisticated structure composed of vast interconnected circuitries that regulate all aspects of learning, memory, emotion, perception and behaviour. Studies estimate that the human brain harbours over a trillion neurons \(10^{12}\) and in excess of a quadrillion synapses \(10^{15}\), reflecting the architectural intricacy of the central nervous system milieu. The ultrastructural morphometric conformation of the neuropil is incredibly diverse, often indicating specialised functional roles (i.e. brain region or structure specific) and adaptive plastic alterations accrued throughout the duration of the lifespan. Of the neuropil compartments, synapses are perhaps the most fascinating structures owing to their dynamic roles in electrochemical neurotransmission, activity-dependent plasticity and the pathogenesis of numerous neurodegenerative diseases.

Synapses, in the simplest sense, are structurally and biochemically unique bulbous swellings located at the distal ends of axons or on branched dendritic segments. The synapse requires two functional partner domains: the presynaptic compartment and the postsynaptic terminal, which form an intercellular adhesive junction facilitating rapid and synchronous transmission of the action potential between neurons. Signal transduction involves the \(\text{Ca}^{2+}\)-dependent fusion and exocytosis of neurotransmitter-rich vesicles from the presynaptic plasma membrane into the synaptic cleft, which bind and activate corresponding receptors expressed on the postsynaptic membrane. This highly efficient signalling cascade is strictly regulated by the presynaptic active zone – a dense filamentous matrix contiguous to the plasma membrane that orchestrates vesicular endocytosis, docking, priming and mobilisation in preparation for release. The active zone is situated in close apposition to the postsynaptic density (PSD), which harbours myriad receptors, ion channels, scaffolding proteins and effector molecules, enabling transient neurotransmitter binding and near-instantaneous \((\sim 5\text{ms})\) signal propagation.
Ultrastructurally, synapses demonstrate distinct morphological markers revealing disparate cardinal functions. Excitatory (asymmetric) synapses are typically located on dendritic spines and exhibit an extensive and illustrious PSD, comprising ionotropic (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), N-methyl-D-aspartate (NMDA), kainate) and metabotropic glutamatergic receptors, PDZ-domain proteins (PSD-95, Discs-large, Zona occludens-1), adhesion molecules and cytoplasmic signalling proteins (20, 21). The unique morphological properties of excitatory synapses enables activation of numerous synergistic inter- and intracellular glutamate-dependent signalling cascades, which have the propensity to instigate dynamic alterations in synaptic transmission efficacy via the induction of long-term potentiation (LTP) or long-term depression (LTD) – processes crucial to learning and memory formation ((22, 23) discussed in further detail in section 1.2.4).

Conversely, inhibitory (symmetric) synapses differ in morphometry and neurotransmission signalling cascades. These synapses appear to primarily occupy the dendritic shaft and inputs surrounding the axonal cell body and demonstrate a significantly less dense PSD coupled with smaller ovoid presynaptic vesicles (10, 21). Despite the PSD expressing scaffolding and adhesion molecules homologous to excitatory synapses, the inherent receptor subtypes anchored to the postsynaptic membrane are functionally regulated by the neurotransmitters gamma-aminobutyric acid (GABA) and glycine (21). Signal transmission by either GABA or glycine does not appear to stimulate analogous LTP- or LTD-dependent synaptic alterations observed at glutamatergic synapses however, evidence suggests these synapses demonstrate a discrete form of inhibitory plasticity mediated by varied ion channels (e.g. Cl⁻ channels), substrates (e.g. endocannabinoids, nitric oxide) and molecular mechanisms (this will not be discussed in further detail but for a comprehensive review see: Castillo et al (24)).
Figure 1: Electron microscopy images demonstrating the unique morphology of excitatory and inhibitory cortical synapses. A. Example image of an excitatory (asymmetric) synapse in the wild-type (WT) mouse cortex. The electron dense PSD is indicated by the white arrow. Note the presence of clear spherical vesicles in the pre-synaptic compartment. B. Example image of an inhibitory (symmetric) synapse in the WT mouse cortex. The white arrow denotes the PSD, which is visibly less dense than the analogous structure located on the excitatory synapse. The pre-synaptic terminal harbours smaller ovoid vesicles. M = mitochondria. Original images provided by Prof. Tom Gillingwater.

1.2.2 Regulation of the synaptic milieu

Diversity in synaptic structure is strongly indicative of function and the two must demonstrate considerable synergy to promote synaptic and neuronal homeostasis. The functionality of both excitatory and inhibitory synapses is profoundly affected by the structural architecture and morphology of the synaptic milieu and aberrant regulation of this spatial organisation through a wide range of insults may result in synaptic demise (see section 1.3). Although there are a plethora of converging cascades mediating trophic factors essential for synaptic stability, the longevity of neurons poses considerable challenges for the maintenance of an intracellular equilibrium. Unlike peripheral cells, which display significant regenerative capacities, the majority of neurons present in the CNS subsist throughout the
duration of the lifespan, requiring proficient synaptic function for decades (25). The average synaptic terminal boasts approximately 300,000 functional proteins (26), comprising multiple copies of those that are essential for compartmental stability, neurotransmission, vesicular-recycling and signalling cascades. Accordingly, this synaptic protein compendium must be dynamically and strictly regulated to ensure optimal functional capacity persists throughout the lifetime of the organism. Although the synaptic compartment demonstrates considerable resilience to intense electrochemical activity, the intracellular environment is often hostile, with regular and significant increases in free Ca\textsuperscript{2+} ions, protons and reactive species that have the propensity to promote protein damage and dysfunction (27). Failure to remove and replenish defective proteins may result in the accumulation of dysfunctional organelles, machinery and macromolecular complexes promoting synaptic destabilisation and concomitant perturbations in the neuronal milieu. Thus, to sustain long-term synaptic structure, function and viability, neurons have developed multiple strategies for the synthesis, delivery and removal of proteins to promote proteostasis in discrete neuronal compartments (25, 27, 28).

1.2.2.1 Axonal transport and local protein synthesis

Regulation of the synaptic proteome is a complex event due to the vast distances (often >100 μm, but up to 1 metre) between synaptic terminals and the protein synthesis machinery located in the cell body (28). A large number of synaptic-specific proteins and compartmental constituents (e.g. mitochondria and vesicles) are pre-assembled in the cell body (29) and transported along the length of the axon to support continuous replenishment of the protein pool and dynamic functional adaptations of the synapse in response to external stimuli (30). Termed “axonal transport”, this active delivery system enables the binding of molecular cargo to motor protein complexes that travel along two types of polarised cytoskeletal structures: microtubules and actin filaments. Microtubules typically facilitate long-distance transport of synaptic cargoes to and from the dendritic shaft via two families of motor proteins: kinesins - which regulate anterograde movement and dyneins - which facilitate retrograde movement (31). Conversely, actin filaments utilise myosin complexes for short-range trafficking and local delivery of cargo to dendritic
spines, where microtubular networks are sparse. The localised expression of the microtubule and actin networks promotes synergistic proteostatic regulation of the synaptic milieu at the spino-dendritic interface, which supports important compartmental functional cascades such as stability, activity and plasticity (31, 32).

Although modulation of the synaptic proteome by axonal transport is an important mechanistic pathway for the delivery of cargo to synapses, the morphological expanse of individual neurons suggests that conveyance of all molecular material would be unfeasible. Axonal transport is a relatively slow process moving at approximately 1.2 µm/second (30), meaning that the duration between the requirement and delivery of essential proteins involved in the dynamic adaptation of the synaptic compartment may be hours, or even days. In conjunction, multiple synaptic proteins display transient half-lives, which suggests that long-range transport would be an unsustainable mechanism for the delivery of functional proteins (30). Thus, it seems probable that synaptic compartments require additional machinery for autonomous regulation of dynamic protein turnover. Indeed, it is now widely recognised that axons and synapses are capable of local protein translation, providing rapid activity-dependent spatial and temporal regulation of proteostasis within the synaptic compartment (25, 27, 28, 33). Axonal segments in apposition to synapses harbour local protein translation machinery, including ribosomes (34) and elongation factors (35), in addition to mRNA transcripts for large numbers of presynaptic proteins (36, 37), which enables near-instantaneous protein expression in response to synaptic activity. Interestingly, discrete populations of synapses from anatomically distinct cortical regions demonstrate varied levels of local translational machinery (33, 38) suggesting that there may be differential mechanistic regulation of the synaptic proteome throughout the cortical architecture dependent on synapse localisation. Although not thoroughly explored, this may have vast implications for the modulation of synaptic proteostasis in ageing and disease states, particularly with regard to selective synaptic vulnerability (discussed further in section 1.3.1). Whilst the underlying mechanisms of local synaptic protein translation remain unresolved, questions persist as to whether synapses also harbour local protein degradative cascades to enable rapid equilibration of intracellular protein concentrations.
1.2.2.2 Protein degradation pathways

Alongside protein synthesis cascades, neurons also possess two major protein degradation pathways – the ubiquitin-proteasome system (UPS) and the autophagic-lysosomal system – for selective removal of damaged cellular components and maintenance of synaptic health ((39, 40) Fig. 2). Although inter-dependent configurations (39), each exhibits discrete functional roles and divergent mechanistic cascades to facilitate targeting of proteins and organelles to the appropriate proteolytic machinery. The UPS typically degrades the majority of short-lived soluble intracellular proteins found at synaptic terminals by selectively tagging and transporting substrates to the 26S proteasome (40, 41). This occurs through a succession of enzymatic reactions regulated by activation (E1), conjugation (E2) and ligase (E3) enzymes, resulting in covalent modification of the target protein via the conjugation of a polyubiquitin chain to acceptor lysine residues (41-43). Once polyubiquitinated, E3 ligases and associated chaperone molecules shuttle the proteins to the proteasome where protein unfolding, proteolysis and deubiquitination pathways yield constituent peptides and free ubiquitin molecules, which are recycled by the neuron ((41, 43, 44) Fig. 2A&B). Although the ubiquitination of proteins is traditionally associated with the UPS catabolic cascade, recent evidence has suggested that ubiquitinated substrates may also be selectively targeted to and degraded by the lysosome (39, 45). Lysosomal-mediated autophagy is the primary macroautophagic mechanism employed by the neuron for the degradation of large structural molecules, insoluble proteins (misfolded, oligomerised, aggregated) and cellular organelles, such as mitochondria (41, 44, 46). The canonical autophagic cascade involves the sequestration of cytoplasmic substrates into a dynamic double-membrane bound vesicle termed an autophagosome, which then fuses with the lysosome for acidic hydrolysis of the engulfed contents ((27, 41, 44) Fig. 2C). The resulting amino acid products are subsequently trafficked from the lysosome to the intracellular amino acid pool for de novo protein synthesis (27), mediated by the cascades described in section 1.2.2.1. Appropriate targeted degradation of local substrates by autophagy is a vital mechanism preventing the accumulation of
synaptotoxic proteinaceous inclusions characteristic of pathological neurodegenerative conditions.

Although the notion of local synaptic degradation has traditionally been a contentious issue, recent evidence has reported the presence of biochemically and functionally unique proteolytic machinery at synaptic terminals (27, 41, 47). Multiple investigations have demonstrated that coordinated proteolysis is a fundamental mechanism facilitating plasticity at cortical synapses (see section 1.2.4) via alterations in the pre- and postsynaptic protein complement (Fig. 2D). Indeed, studies examining the role of the UPS in the regulation of synaptic transmission report activity-dependent translocation of the proteasome to the postsynaptic compartment mediated by the binding of glutamate to NMDA receptors. The proteasome locally acts to promote the ubiquitination and internalisation of transmembrane AMPA receptors (48), which concomitantly modulates synaptic efficacy by inducing LTD cascades ((41, 48, 49) discussed in section 1.2.4). Internalisation of AMPA receptors and induction of LTD may be modulated by application of proteasome inhibitors (49), suggesting that the UPS is actively regulating the strength of synaptic transmission via the ubiquitination of intracellular and transmembrane substrates ((41) Fig. 2D). Similarly, acute neurotransmission activity also facilitates the transient upregulation of autophagosomes and lysosomes in the pre- and postsynaptic compartments, which promotes the rapid engulfment of selected internalised AMPA receptors (27, 48, 50) and rejuvenation of the active zone vesicular pool (27, 42). Application of autophagy inhibitors to neurons in vivo elicits reductions in synaptic vesicle number, neurotransmission and induction of plasticity cascades (42, 51), suggesting that local autophagic regulation of the synapse is a fundamental cascade bolstering synaptic activity and function (Fig. 2D).

The resolute equilibration of synaptic protein synthesis and degradation is critical for the maintenance of synaptic health. Investigations have demonstrated that autonomous temporal and spatial modulation of the synaptic proteome by local machinery facilitates alterations in synaptic structure, function and efficacy, which may synergistically regulate the molecular mechanisms mediating memory
formation. However, how these cascades are autonomously regulated or how advancing age may impede synaptic proteostasis remains elusive.
1.2.3 Synaptic mitochondria

Mitochondria are present in nearly every mammalian cell and numbers range from several hundred to >1000 dependent upon cellular type and basic functional requirements (52, 53). Although typically believed to be symmetric ovoid organelles, mitochondria exhibit significantly varied morphologies dependent upon tissue, subcellular localization and the biochemical properties of the intracellular milieu (54,
in order to functionally promote homeostatic equilibration. Despite the varied shape and size of the organelles, the configuration of the membranous structures encompassing the intrinsic molecular machinery remains relatively consistent. Mitochondria harbor a double-membrane arrangement, which forms four biochemically discrete compartments: the outer-membrane, the inter-membrane space, the inner membrane and the matrix (52, 53, 55). Of central importance is the inner membrane, which houses the enzymatic complexes (I-V) of the respiratory chain (52, 53, 55) and enables the generation of adenosine triphosphate (ATP) via the process of oxidative phosphorylation to fuel virtually every cellular process (Fig. 3). The respiratory chain is a structurally and biochemically sophisticated network under dual genetic control from nuclear (nDNA) and mitochondrial DNA (mtDNA) (52, 56), which promotes the systematic assembly of the complexes via integration of the 13 mtDNA encoded proteins and >100 nDNA polypeptides critical for function. Through this arrangement, the proteins act synergistically to generate a proton motive force across the inner membrane by the sequential reduction of electrons in complexes I-IV, promoting a net accumulation of protons outside the inner membrane (57). The flow of protons back into the mitochondria via the F₁/F₀ ATP-synthase (complex V) drives the synthesis of ATP (52, 53), which is transported to the cytosol by ADP/ATP carriers (58). The synthesis and dissemination of ATP by the mitochondrial machinery supports a multitude of functions in the CNS including neurotransmission cascades, synaptic vesicle endocytosis and release, trafficking of intracellular cargo and maintenance of essential signaling pathways (59, 60).

Figure 3: The mitochondrial respiratory chain. Image adapted from Sazanov, 2016 (61).
Mitochondria are incredibly dynamic organelles and exhibit continuous bi-directional trafficking along the neuronal cytoskeleton via actin cables and microtubule motors (60). Binding of mitochondria of the appropriate size and shape to the cytoskeleton (facilitated by fission and fusion proteins) is achieved through the motor-adaptor-receptor complexes kinesin (KIF5) and dynein, which regulate anterograde and retrograde motility and ensure targeted trafficking of the mitochondria in response to biochemical fluctuations in the intracellular milieu (54, 60, 62). The transport of mitochondria appears to be regulated by synaptic activity and the organelles are rapidly redistributed to the pre- and postsynaptic compartments in response to two signals: ATP depletion and elevations in intracellular Ca\(^{2+}\) (60, 62). Accordingly, recruitment and docking of mitochondria to the synapse enables replenishment of the ATP pool and clearance of free Ca\(^{2+}\) ions - processes required for homeostatic control and the induction of synaptic plasticity (59, 62). Interestingly, mitochondria localized to the synaptic compartment are biochemically distinguishable from those organelles located in the axonal milieu (63). Investigations document that the synaptic mitochondrial subpopulation displays unique enzymatic (63), calcium buffering (64, 65) and antioxidant properties (66), which are considered to occur in response to the environmental conditions at the synapse. Of particular interest are reports that there is significant subcellular heterogeneity with regards to calcium handling mechanisms. Synaptic mitochondria exhibit increased susceptibility to progressive Ca\(^{2+}\) overload versus non-synaptic mitochondria and rapidly initiate the catastrophic permeability transition pore, which promotes loss of mitochondrial membrane potential, rupture of the outer membrane and ultimately mitochondrial demise (64). Although experimentally undetermined, the limited Ca\(^{2+}\) buffering capacities of synaptic mitochondria may facilitate perturbations in synaptic stability during ageing and disease, as the functional demands of the active synapse are not being met. The cause of such heterogeneity remains elusive, however various studies have suggested that the maladaptive properties of synaptic mitochondria may occur due to the failure of retrograde transport (66), promoting “ageing” of the synaptic mitochondrial subpool and
concomitant dysfunction (64, 67). Despite this, the mechanistic pathways mediating such events have not been well characterised.

Recently, *in vitro* investigations have begun to examine subcellular heterogeneity in the mitochondrial proteome. Indeed, there are reports of synaptic mitochondria harbouring discrete proteomes, with varied expression of the proteins comprising the respiratory complexes, fission and fusion, Ca\(^{2+}\) buffering and mtDNA maintenance (68), providing insights into the differential regulation of unique mitochondrial populations. Although these data are indubitably important with regards to biochemical adaptation of discrete mitochondrial populations, the use of cell lines may not faithfully recapitulate a biological system. As illustrated throughout this section, it is well established that mitochondria are highly dynamic organelles, consistently altering their morphological and functional parameters, as well as their protein complement, to adapt to the surrounding cellular milieu. Thus, the expression of the synaptic and non-synaptic mitochondrial proteomes will likely be highly responsive to the *in vitro* environment. The current knowledge concerning differential mitochondrial protein expression in the mammalian CNS remains in its infancy despite numerous efforts to elucidate the biochemical composition of discrete organellar subpopulations. Although it is probable that synaptic mitochondria contribute to adverse age-dependent alterations, further studies are required to delineate the functional implications of differential mitochondrial protein expression *in vivo*, particularly upon the synaptic architecture.

1.2.4 Synaptic Plasticity

As alluded to in *sections 1.2.1 and 1.2.2*, synaptic plasticity is the putative biological substrate underpinning the neural basis of learning and memory. It has been well established that glutamatergic synapses, particularly those resident to the hippocampus, demonstrate activity-dependent modifications in synaptic transmission efficacy (22, 23). This “plasticity” is capable of promoting the strengthening (long-term potentiation (LTP)) or weakening (long-term depression (LTD)) of synaptic
connections - effects dictated by the parameters of neural stimulation, voltage-dependent activation of the NMDA receptor and calcium-mediated signalling.

LTP and LTD are incredibly sophisticated molecular cascades, which remain somewhat equivocal with regards to the biochemical mechanisms governing specific mnemonic processes. Although a holistic mechanistic model of synaptic plasticity has not yet been established, genetic and pharmacological investigations have confirmed that a number of properties must be satisfied for the induction, expression and maintenance of LTP and LTD (23, 69-71). Crucially, to induce plastic modifications at the synapse, the voltage-gated NMDA receptor channel must be opened to allow the permeation of Ca$^{2+}$ ions into the postsynaptic terminal, promoting signal transduction, functional changes in channel conductance and sustained alterations in synaptic strength. Although both LTP and LTD are mediated by the NMDA receptor, it is the amplitude of the arriving action potential that dictates whether potentiation or depression of the synapse occurs (22, 23, 69, 72). LTP induction typically commands high frequency tetanic stimulation for opening of the NMDA receptor pore and the subsequent strengthening of synaptic connections (22). At resting membrane potentials, the NMDA receptor harbours a Mg$^{2+}$ ion blockade, preventing the permeation of cations through the channel, however the arrival of a depolarising stimulus of sufficient amplitude is capable of surmounting the blockade, expelling the Mg$^{2+}$ ions from the NMDA pore and enabling maximal influx of Ca$^{2+}$ ions into the postsynaptic terminal (73). Thus, the NMDA receptor is often referred to as a molecular coincidence detector as opening of the pore and induction of LTP requires two events to occur concurrently: i) the release and binding of glutamate to postsynaptic receptors and ii) strong depolarisation of the postsynaptic membrane to enable expulsion of blockading Mg$^{2+}$ ions from the NMDA receptor pore and permeation of Ca$^{2+}$ ions (23, 73). Similarly, LTD induction also requires an increase in postsynaptic Ca$^{2+}$ concentration, however this is typically achieved by repeated low frequency stimulation of the presynaptic neuron (69-72). The NMDA receptor Mg$^{2+}$ ion blockade is not absolute, even at resting membrane potentials, and modest activation of the receptors invoked by a
low amplitude depolarising stimulus promotes sufficient influx of $\text{Ca}^{2+}$ ions for transduction of the LTD signal without necessitating $\text{Mg}^{2+}$ expulsion (74).

Although LTP and LTD are mutually exclusive events, occurring on a divergent spectrum of synaptic plasticity, both demonstrate calcium-dependent signal transduction mechanisms that facilitate enduring modifications in synaptic efficacy (75, 76). Cyclic AMP (cAMP), calcium calmodulin-dependent kinase 2 (CaMKII), protein phosphatase 1 (PP1) and calcineurin have all been championed as fundamental regulators of plasticity owing to their prevailing role in various mechanistic phosphorylation cascades within the pre- and postsynaptic milieu (77-79). Cyclic AMP and CaMKII mediate the $\text{Ca}^{2+}$-dependent phosphorylation of AMPA receptors, promoting enhanced receptor trafficking and insertion at the postsynaptic membrane in addition to increased single channel conductance (80), which supports the long-term maintenance of synaptic potentiation. Conversely, lower concentrations of intracellular $\text{Ca}^{2+}$ activate the phosphatases PP1 and calcineurin, which dephosphorylate AMPA receptors promoting receptor internalisation, reduced channel efficacy and depotentiation of the synapse (78, 79). In conjunction with post-translational modifications at AMPA receptors, sustained activation of LTP/LTD signalling cascades promotes cAMP to function as a mediator between the synaptic and nuclear compartments, initiating alterations in gene transcription and local protein synthesis and degradation via the cascades discussed in section 1.2.2 (23, 69).

The assimilation of these events and the corresponding morphological alterations occurring at the pre- and postsynaptic specialisations (i.e. changes in spine and active zone density and numbers (81)), are considered to orchestrate the mechanisms of memory storage and longevity. How the biophysical properties of the pre- and postsynaptic terminals alter during advancing age and contribute to impairments in cognitive capacity will be discussed in section 1.3.2.
1.3 The ageing cortical synapse

Widespread neuronal loss was traditionally considered to be the anatomical correlate of age-related cognitive decline. Early investigations into mammalian cortical ageing reported significant reductions in brain weight and neuronal density (82-85) resulting in a broad consensus that the degeneration of neuronal populations was an inevitable component of normal ageing. However, this hypothesis has since been refuted owing to the application of modern stereological techniques (86-88). It is now widely accepted that typical age-related alterations in cognitive capacity are associated with subtle vicissitudes in the synaptic milieu as opposed to gross cellular loss, which is characteristic of pathological conditions (4). Studies increasingly report that perturbations in various dendritic modalities, including arborisation, spine number, volume, density and stability (4, 5, 89, 90), alterations in synaptic firing patterns (4, 5) and increases in free radicals (91, 92) systematically encumber cognition during advancing age. Despite this, the molecular precursors bolstering the documented age-dependent morphological and functional shift remain elusive and studies are only now beginning to unravel how discrete organelles, proteins and signalling cascades may hierarchically or synergistically attenuate synaptic function.

1.3.1 Brain regional vulnerability of discrete synaptic populations

Discrete neuroanatomical regions of the cortical architecture harbour disparate populations of synapses that demonstrate significant heterogeneity with regards to advancing age (3-5, 93, 94). This notion of “selective synaptic vulnerability” has been the focal point of a multitude of morphological studies investigating age-related cognitive decline (94-98), which have often provided tentative conclusions as to how this phenomenon may be mediated. Despite this, the data have enabled a thorough structural characterisation of specific subsets of synapses that appear to demonstrate heightened susceptibility or resistance to age-related alterations and/or loss. Of particular interest are the prefrontal cortex (PFC) and hippocampus, which are reportedly the most vulnerable brain regions during ageing, and the occipital cortex, which demonstrates relative resistance to age-dependent synaptic alterations ((3, 94,
99) the PFC will not be discussed in detail here - please refer to Morrison & Baxter, 2012 (4) for an extensive review.

1.3.1.1 Morphological alterations in ageing hippocampal synapses

Anatomical studies in human patients (100), rhesus macaques (101) and rodents (102) consistently describe significant age-dependent morphometric alterations in hippocampal synaptic number and density, which appear to correlate with cognitive function (4, 13). The perforant path – projecting from the entorhinal cortex to the outer molecular layer of the dentate gyrus (Fig. 4A) – is exceedingly vulnerable to age-related alterations (103-105) and exhibits complex changes in multiple morphological parameters, including pre- and postsynaptic coupling, in the ageing rhesus macaque (4, 103, 104). Light and electron microscopy studies examining the perforant path have reported a significant age-dependent preferential loss of axodendritic synapses from the outer molecular layer of the non-human primate (NHP) dentate gyrus, but preservation of the ultrastructurally distinct axospinous synapses from the same region (95). Despite this, reductions in total numbers of axodendritic synapses do not correlate with hippocampal-mediated cognitive impairments (106). Instead, it has now been proposed that subtle alterations in the properties the excitatory axospinous synapses furnishing the outer molecular layer facilitate age-related reductions in cognitive capacity. Indeed, investigations characterising pre- and postsynaptic terminal coupling in young and old NHPs describe a 100% increase in synaptic terminal dissociation (resulting in non-synaptic boutons) in addition to a decrease in the number of perforated synapses and multisynaptic boutons in the aged macaque ((106) Fig. 4B). This morphological shift is indicative of spine retraction, or, alternatively, a reduction in long-term spine retention, suggesting that the aged hippocampus may not be capable of adequately maintaining a significant volume of synaptic connections. Interestingly, the number of non-synaptic boutons robustly correlates with cognitive impairment on hippocampal-dependent behavioural tasks (106), indicating that the stability of pre-established complex synaptic connections in the outer molecular layer, and other sub-regions of the dentate gyrus, supports the maintenance of cognitive capacity (Fig. 4C).
The anatomical organisation of the primate hippocampus is relatively well preserved in rodents (Fig. 4A) and reports consistently document that rats, like humans and NHPs, exhibit hippocampal-dependent cognitive impairments. Investigations examining the morphological correlates associated with alterations in rodent cognition have identified perturbed synaptic structures in homologous regions to the NHP – primarily, an age-dependent selective loss of synapses from the outer molecular layers of the dentate gyrus (4, 107). Although the loss of these axodendritic synapses in the NHP does not correlate with cognitive capacity, aged rats demonstrate significant memory impairments, likely mediated by the loss of both axodendritic and axospinous synaptic subtypes (102, 107). Further evidence utilising immunolabelling techniques coupled with electron microscopy indicates that aged rats also harbour significant reductions in the number of synaptic terminals in the hippocampal CA3 circuitry versus younger animals and, strikingly, the loss of these distinct synapses directly correlates with spatial learning deficits (108, 109). Moreover, despite morphological preservation of the CA1 circuitry in aged humans and NHPs, rats display subtle vicissitudes in the synaptic milieu comprising the regional CA1 architecture. Analyses report significant reductions in postsynaptic density area on perforated synapses (an approximate 27-44% loss), which appears to mediate spatial learning deficits (110). Although perturbations in the human CA1 synaptic population is indicative of pathogenesis, the natural age-dependent deterioration of this circuitry in the rat may provide insight into conserved cognitive pathways and functional mediators of mammalian synaptic stability.
Figure 4: Alterations in hippocampal synaptic morphology correlate with age-related cognitive impairment. A. Age-dependent changes in the hippocampus. Schematics depict hippocampal circuitry and alterations in the density of perforant path spines between young and aged animals (EC = entorhinal cortex; DG = dentate gyrus). B. Schematics illustrating age-dependent morphological alterations in hippocampal synaptic subtypes. Aged animals display an increase in the number of non-synaptic boutons and reductions in multi-synaptic boutons versus young. C. A significant inverse correlation exists between the percentage of detectable non-synaptic boutons in the dentate gyrus and the average accuracy on the hippocampal-dependent delayed non-matching-to-sample (DNMS) cognitive test. SSB = single synaptic bouton; NSB = non-synaptic bouton; MSB = multi-synaptic bouton. Panel A adapted from Fan et al, 2017 (7); panels B & C adapted from Morrison & Baxter, 2012 (4).

The age-dependent selective vulnerability of the hippocampal synaptic milieu evidently bolsters reductions in cognitive capacity. Although it is now well established that morphological alterations in complex synapses associated, including a decline in multi-synaptic boutons and previously potentiated perforated synapses
(4), promote impairments in memory storage, retention and retrieval, the molecular mediators orchestrating this preferential loss remain elusive. There is a substantial requirement for well-executed biochemical investigations to examine the temporal alterations occurring in hippocampal synapses and determine how molecular networks may be promoting perturbed structure and function. Such studies may enable the development of therapeutic strategies promoting the stability of these receptive compartments, which is of paramount importance for preservation of cognitive capacity during advancing age.

1.3.1.2 Morphological alterations in ageing occipital cortex synapses
Although there has been significant scientific focus on the selective synaptic vulnerability of the hippocampal formation, few studies have investigated the age-dependent trajectory of occipital cortex synapses despite the reported preservation of structural and functional stability throughout the duration of the lifespan (3, 94, 99). Indeed, studies examining temporal alterations in the NHP occipital cortex synaptic milieu report no age-dependent morphological changes (Fig. 5B&C), including preservation of synaptic number, spine density and dendritic arborisation (3, 94, 99, 111). In addition, gross anatomical investigations utilising magnetic resonance imaging (MRI) document no significant volumetric changes associated with advancing age in the occipital cortex, with area and cortical thickness displaying quantitatively similar measurements in both young and old humans (Fig. 6), as well as rhesus macaques (94, 112, 113). Coupled with the outlined stereological studies, there is also recent behavioural evidence describing that performance on visual discrimination tasks is consistent throughout the lifespan in the rhesus macaque, which suggests functional preservation of the region during ageing (99). Furthermore, there are extraordinary reports documenting that even in patients with advanced neurodegenerative conditions such as AD, the occipital lobes remain relatively unaffected, with sparse pathological tau inclusions (114, 115) and preservation of the synaptic architecture (116-119) and total cortical volume (120, 121).
**Figure 5: Morphological preservation of the occipital cortex during ageing.**

A. Asterisks mark the anterior margin of the occipital cortex in the rhesus macaque brain. B&C. Unbiased stereologic analyses demonstrate preservation of total cortical volume and total neuronal number in the occipital cortex during ageing. Scale bar = 1cm. Image adapted from Hof et al, 2002 (94).

The age-dependent resistance of the synaptic populations furnishing the occipital cortex is certainly intriguing and, to our knowledge, there have been no investigations examining the biochemical cascades orchestrating this inherent neuroprotection. Despite this, a recent study has begun computationally characterising the dendritic differences between vulnerable PFC neurons and resistant occipital cortex neurons (122). Interestingly, the occipital cortex exhibits significantly reduced dendritic complexity, with fewer spines, smaller dendritic arbours and conserved cortical connectivity (99, 122), which may promote resistance to age-dependent alterations via functional mechanisms yet to be determined. Elucidating the morphological and molecular correlates of occipital synaptic protection may be fundamental in understanding the selective vulnerability of discrete populations of synapses and recapitulation of such physiological cascades may provide a therapeutic strategy to ameliorate adverse synaptic alterations in the ageing hippocampus and PFC, promoting cognitive health.
Figure 6: Differentially vulnerable brain regions demonstrate discrete volumetric alterations during ageing in human patients. Each point indicates the calculated volume of the occipital cortex (A.) or the hippocampus (B.) in a single patient from MRI images. The hippocampus exhibits volumetric decreases after 60 years of age whereas the occipital cortex volume remains relatively preserved. Image adapted from Hedden & Gabrieli, 2004 (112).

1.3.2 Synaptic plasticity in the ageing brain

As previously discussed, synaptic structure and function are intricately intertwined, exhibiting significant synergy to promote compartmental stability. It is well established that selected hippocampal synapses display morphometric shifts during ageing (see section 1.3.1.1), which is highly suggestive of concomitant modifications in functional plasticity cascades. Indeed, investigations examining the molecular mediators of hippocampal plasticity have documented a plethora of age-dependent changes in critical properties believed to modulate cognition including Ca\(^{2+}\) homeostasis, NMDA receptor expression and LTP induction threshold (5, 103, 123).

Although numerous electrophysiological properties remain constant through the duration of the lifespan (e.g. resting membrane potential, action potential threshold (5)), a salient feature of rodent hippocampal ageing is an increase in Ca\(^{2+}\) conductance in the CA3 and CA1 circuitry, promoting K\(^{+}\)-mediated increases in the magnitude of the after-hyperpolarising (AHP) phase of the action potential (5, 123, 124). The amplification and extended duration of the AHP in selected hippocampal neurons impedes cell excitability and synaptic plasticity by preventing the
subsequent generation of an action potential and attenuating firing frequency of the synapse (4, 5, 123). This phenomenon is believed to be mechanistically regulated by age-dependent elevations in intracellular \( \text{Ca}^{2+} \) via increased expression of L-type \( \text{Ca}^{2+} \) channels and alterations in \( \text{Ca}^{2+} \) buffering and extrusion machinery (this is briefly discussed in section 1.2.3). Disruptions in intracellular \( \text{Ca}^{2+} \) homeostasis during advancing age have been widely reported and are frequently associated with perturbations in the induction of LTP and LTD cascades. As outlined in section 1.2.4, the potentiation and depression of synaptic compartments is \( \text{Ca}^{2+} \)-dependent and alterations in ion equilibration may diminish neuronal capacity to induce such plastic modifications. Indeed, elevations in postsynaptic intracellular \( \text{Ca}^{2+} \) concentrations in the aged rat dentate gyrus and CA3 promote a functional shift in plasticity, with neurons preferentially inducing LTD over LTP due to \( \text{Ca}^{2+} \)-dependent modifications of the synaptic potentiation threshold (125, 126). Additionally, moderate increases in intracellular \( \text{Ca}^{2+} \) levels are reported to promote LTD in aged animals by stimulating calcineurin activity, which inhibits NMDA receptor opening via dephosphorylation cascades (127). Further reports of age-related reductions in LTP document the preferential loss (30%) of an essential NMDA receptor subunit (NR1) in the NHP perforant path (specifically the outer molecular layer of the dentate gyrus), which has been suggested to mediate cognitive deficits during advancing age due to disruptions in LTP-dependent learning and memory processes (103).
Collectively, evidence supports the notion that focal disruptions in Ca\(^{2+}\) homeostasis promote perturbations in synaptic plasticity and cognitive capacity by mechanistically altering cell excitability via the neuronal AHP and potentiation threshold. Investigations have begun to describe that restoration of “young” intracellular Ca\(^{2+}\) concentrations in old animals facilitates learning and memory through modulation of the AHP and activation of LTP cascades (128, 129), suggesting that age-related cognitive decline is, in part, due to alterations in the plastic properties of the synapse. Despite this, the molecular mediators triggering the initial dysfunction in Ca\(^{2+}\) homeostasis remain unresolved. Temporal examinations of Ca\(^{2+}\) sequestration and extrusion properties up- and downstream of neurotransmission cascades may elucidate the molecular machinery contributing to the age-dependent vulnerability of the synaptic milieu. Furthermore, investigations exploring how alterations in functional plasticity pathways may be hierarchically or synergistically driving morphological changes in selected synaptic populations are required for amelioration of both age-dependent structural and functional phenotypes.

1.3.3 Temporal regulation of the synaptic proteome

Though the studies described have provided comprehensive insights into age-dependent modifications in synapse morphology and reductions in synaptic efficacy, few have examined the global molecular alterations that may be bolstering these...
events. Investigations are now beginning to utilise unbiased quantitative “–omic” approaches to discern how dynamic modulations in transcript or protein expression may mechanistically mediate structural and functional perturbations in the aged synaptic milieu. Indeed, studies characterising temporal expression changes in the rat hippocampal synaptic proteome using two-dimensional difference gel electrophoresis (2D-DIGE) and MS/MS techniques, report significant and synchronous reductions in a multitude of proteins regulating synaptic transmission and vesicle dynamics during advancing age (130, 131). Furthermore, microarray investigations assessing the expression of 340 selected synaptic transcripts describe an age-dependent downregulation of genes mediating neurotransmission cascades, PSD scaffolding and cell adhesion in multiple cortical regions (132). Together, the data suggest that the progressive loss of critical proteins regulating the properties of neurotransmission during ageing likely modulates the reported alterations in electrophysiological properties and thus may represent a molecular precursor to perturbed plasticity and concomitant cognitive impairment.

However, despite the outlined “–omic” studies providing protein expression data consistent with reported alterations in synaptic transmission efficacy, the use of outdated methods (2D-DIGE) has hindered the number of protein identifications and quantitation of low-abundance proteins (some of which may play a fundamental role in regulating synaptic stability and/or vulnerability during ageing). The application of modern mass spectrometry techniques to isolated regional synaptic compartments will be fundamental in ascertaining the global temporal changes associated with selective alterations in structure, function and stability of the synapse. Improved methodologies and optimised workflows will promote examination of the dynamic processes facilitating age-dependent cognitive impairment and enable identification of the mechanistic cascades orchestrating the functional demise of the synapse.
1.4 Models of ageing

1.4.1 Non-human primates

Modelling human ageing is a complex and challenging notion. Ageing research has been somewhat hindered by the lack of reliable models that faithfully recapitulate the array of physiological alterations occurring in the aged human patient. Numerous investigations have adopted rodent models in an attempt to decipher the biochemical substrates bolstering neuronal instability, however this approach often lacks sufficient translational impact due to the documented differences in fundamental processes considered relevant to the ageing trajectory. Rodents possess limited lifespans and thus the natural development of common age-related comorbidities (e.g. diabetes, sarcopenia, atherosclerosis) does not appear to occur (133). Although this may be convenient for a laboratory study, a multitude of recent investigations have provided evidence that the presence of a comorbid disorder may attenuate neuronal and synaptic stability, contributing to the development of age-related cognitive decline in non-human primates and human patients (134-137). Furthermore, evolutionary divergence of the primate cortex has promoted functional neuronal alterations that are uniquely primate (4) and studies documenting rodent biological brain ageing are not necessarily representative of the complex processes occurring in the human patient, particularly with regards to selective synaptic vulnerability (138). Thus, in order to tease apart human age-related cognitive decline, non-human primates provide some advantages. Non-human primates are phylogenetically closer to humans and possess distinctly primate morphological, endocrine, behavioural, and cognitive traits (4, 94), in addition to an increased lifespan (Fig. 8), which may provide data uniquely relevant to human ageing.

Of particular importance is the rhesus macaque (*Macaca mulatta*) – an old world monkey demonstrating an estimated 93% genetic similarity with humans (139). Rhesus macaques are the most widely utilised NHP in ageing research due to their practical size (5-10kg), protracted captive lifespan of 25-30 years (Fig. 8) and capacity for extensive cognitive testing (3, 94, 133). Importantly, laboratory rhesus
Macaques are outbred, offering biologically relevant genetic diversity between animals, which is akin to that of humans (140). Indeed, morphological and behavioural studies in macaques demonstrate that age-dependent neuronal alterations and concomitant cognitive impairments occur in only a subset of animals, mirroring the epidemiological incidence of such impairments in the human population (103, 141, 142). However, unlike humans, environmental, dietary and social interactions may be controlled (133), providing a structured platform with which to dissect the physical and physiological mechanisms governing the effect of advancing age on the neuronal milieu.

Despite ageing rhesus macaques exhibiting cellular and behavioural alterations analogous to human patients, including selective synaptic loss, alterations in neuronal biophysical properties and impairments in short-term memory and executive function ((3, 4) see section 1.3), NHPs do not suffer from age-related neurodegenerative diseases (e.g. AD (3, 94, 103)). Although the aged macaque displays varying levels of β-amyloid (Aβ-40) deposits throughout the brain accompanied by dystrophic phenotypes in the surrounding neuronal architecture, the localisation and frequency of plaques correlates solely with increasing age, not cognitive status (3). Interestingly, neurofibrillary tangles - the pathological drivers of cognitive dysfunction in AD (116) - appear to be uniquely human, with no reports of such substrates in even the oldest macaque brains, despite the expression of homologous tau protein (3).

NHPs provide a uniquely relevant model to assess how the trajectory of human cortical ageing may be mediated. Although the age-dependent structural alterations occurring in the neuronal milieu and concomitant behavioural changes have been extensively characterised in the rhesus macaque, the molecular substrates governing these morphological adaptations remain elusive. Biochemical investigations examining the mechanistic cascades regulating such events in the NHP will harbour numerous benefits, as the effect of age may be assessed independently of the pathological processes governing discrete forms of dementia.
Figure 8: Rhesus macaque survival curve. Graph depicts survival of animals in captivity and the reported stages of ageing. Onset of cognitive decline typically appears between ages 18-22. Data obtained from Moss et al, 1999 (143).

1.4.2 Drosophila melanogaster

Though the use of NHP tissues in ageing studies provides invaluable data regarding the molecular mechanisms orchestrating human synaptic ageing, the generation of such models is often timely and requires significant laboratory space as well as expertly trained individuals. Thus, alternate models of ageing, such as Drosophila melanogaster, may offer additional insights into the spatiotemporal regulation of synaptic biochemistry during advancing age. The development of sophisticated tools for use in Drosophila melanogaster models has offered pioneering strategies enabling the characterization of a multitude of genes, proteins and biochemical cascades regulating the stability of the nervous system. Although from an evolutionary perspective Drosophila may appear to display significant phylogenetic distance from humans, numerous fundamental mammalian biological and neurological pathways have been shown to demonstrate conservation in the fruit fly (144). Additionally, sequencing of both the primate and Drosophila genomes has indicated that up to 75% of human disease related genes exhibit a functional ortholog.
in the fly (145, 146), thus providing a reliable model that rapidly and faithfully recapitulates aspects of mammalian pathologies.

The use of *Drosophila* models in the research environment is an attractive prospect – not only for the documented mammalian genetic homology, but also for the arsenal of innovative tools, stereotyped organization of the nervous system and timely genesis of adult flies. The *Drosophila* life cycle is a dynamic 10-day event consisting of several ephemeral developmental stages, resulting in larval metamorphosis and the eclosion of adult flies (Fig. 9). Adult females have the ability to lay hundreds of fertilized eggs over the duration of several days (146, 147), with first instar larva hatching from the egg after completion of embryogenesis. Maturation of the larva continues through the second and third instar stages before the animals pupariate for the initiation of histolytic processes (147), which concomitantly stimulates the generation of adult anatomical structures. Once complete, adult flies eclose from the pupal casing in a circadian-rhythm dependent manner (148), allowing the life cycle to recommence.
Figure 9: The *Drosophila* life cycle. *Image obtained from Creative Diagnostics.*

The swift generation of flies provides obvious advantages with regards to the collection of experimental data and many *Drosophila* based laboratories selectively utilize high throughput genetic screens to delineate the functions of hundreds of genes over relatively short time frames. Primarily, research focuses on two methods of manipulation to examine genetic function: loss of function (LOF) and gain of function (GOF). Suitably named, loss of function studies serve to partially or wholly abolish candidate function primarily by introducing LOF mutant alleles or RNA interference (RNAi) constructs (147). Conversely, investigations interrogating gain of function properties typically employ GOF mutant alleles or targeted over-expression constructs to assess hypermorphic effects of the candidate (147). Fortunately, nearly every *Drosophila* gene may be manipulated to provide targeted LOF and/or GOF genotypes through the insertion of P-element transposons into the fly genome during embryogenesis. Although the classification of P-element lines is
vast, two particular collections are of relevance to this project: Gal4/Upstream Activating Sequence (UAS) and RNAi.

The Gal4/UAS system has become an indispensable tool for elucidating gene function since its introduction in 1993 by Brand & Perrimon (149). The sophisticated genetic approach allows directed expression of target genes in a tissue and cell-specific manner by utilizing a bipartite arrangement consisting of driver (Gal4) and target (UAS) elements maintained on discrete parental lines (147, 149, 150). Importantly, the Gal4 and UAS constructs remain transcriptionally silent in the parent lines (149), due to the presence of solely the driver or target elements, which supports the sustained viability of the organisms. It is only with generation of offspring by crossing these two lines that spatiotemporal activation of the target gene occurs, as the progeny harbour both the Gal4 driver and UAS target elements (Fig. 10). The use of UAS-RNAi strategies has become a prevailing approach employed by laboratories to assess genetic loss of function. UAS-RNAi constructs are available for almost every Drosophila gene (147), promoting targeted Gal4-dependent knock-down of selected candidates in particular tissues for examination of mechanistic cascades regulating phenotypic presentations in vivo. Although Drosophila and numerous other organisms do not inherently express the endogenous saccharomyces cerevisiae protein Gal4 (149, 150), transgenic insertion of the gene into selected tissues or cells provides functional expression in vivo, allowing meticulous control of UAS-linked gene activation and delineation of genetic function. Superfluous to the elegance of the Brand & Perrimon’s system (149) was the identification of temperature-dependent activity of Gal4, which allows for increased versatility with use of the approach, including tailored expression of the target transgene through specific stages of development (150).
**Figure 10: Schematic depicting the Gal4/UAS system.** The driver (Gal4) and target (UAS-Gene X) lines are maintained separately. Crossing of the lines generates offspring harbouring both components of the Gal4/UAS system promoting tissue-specific transcription of the gene of interest. *Image adapted from Brand & Perrimon, 1993 (149) and Roote & Prokop, 2013 (147).*

Application of the tools described to study the mechanistic regulation of the *Drosophila* nervous system has provided fundamental insights into the molecular arrangement and function of the mammalian neuronal milieu. Much of the knowledge concerning biochemical modulation the nervous system has been derived from studies utilizing the third instar larva neuromuscular junction ((NMJ) Fig. 11A). The larval NMJ is an excellent model system to unravel the molecular mechanisms underpinning synaptic structure, function and plasticity (151) due to its simple stereotypic morphology, easily accessible musculature and distinguishable subtypes of synapses ((152) Fig. 11). The body wall musculature contains 32 motor neurons in each abdominal hemisegment (152) with each exhibiting distinct arborisation patterns and variations in expression of type Ib, Is II and III synaptic boutons. The majority of these boutons are glutamatergic and harbour contiguous invaginated post-synaptic membranes (sub-synaptic reticulum (SSR)) (153, 154),

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53
which display significant homology to those found in the mammalian central nervous system. Neurotransmission between these connections has the propensity to invoke plastic modifications (151, 153, 155), providing physiologically relevant mechanistic insights into proteins regulating the structure and function of the synapse in mammalian cognition (155).

**Figure 11: The larval body wall musculature and synaptic bouton subtypes.** A. Left panel: larval fillet preparation displaying the body wall musculature at 4x. Right panel: schematic illustrating the stereotypic morphology of larval abdominal hemisegment A3. The frequently examined muscle 12 and 13 NMJ is highlighted in red. B. Panel exhibits morphometry of discrete subtypes of synaptic boutons expressed at larval NMJs. SSR – subsynaptic reticulum; NT – neurotransmitter. *Panel B adapted from Menon et al, 2013 (152).*
1.5 Experimental Aims

1.5.1 Overarching aims

i) Devise a reliable and replicable unbiased quantitative methodological workflow for examination of the spatiotemporal molecular alterations occurring in the synaptic milieu

ii) Utilise the methodological workflow to establish species-specific synaptic signatures during advancing age

iii) Identify proteins, pathways and biochemical cascades that may have the propensity to alter synaptic stability

iv) Determine if identified candidates modulate the synaptic architecture in vivo

v) Assess the similitude of spatiotemporal molecular alterations occurring between species to determine whether analogous biochemical cascades regulate synaptic ageing in the rodent, non-human primate and human patient

1.5.2 Chapter aims

Chapter 3: Characterise the proteomes of rodent mitochondria derived from discrete subcellular compartments and demonstrate how varied protein expression may modulate the stability of the synaptic milieu.

Chapter 4: Examine the temporal alterations occurring in the rodent synaptic and non-synaptic mitochondrial proteomes and identify candidates capable of promoting synaptic vulnerability during advancing age.

Chapter 5: Isolate and examine the temporal proteomic profiles of synapses derived from two differentially vulnerable NHP brain regions and demonstrate how age-dependent preferential synaptic loss may be mechanistically regulated.

Chapter 6: Utilise synapses purified from analogous human patient brain regions to describe dynamic alterations in the synaptic proteome and how these may dictate age-dependent vulnerability of the hippocampal synapse.
Chapter 2. Materials and Methods

This section describes the general methodologies utilised in Chapters 3-6. For detailed experimental procedures please refer to the specific methodological subsections associated with each chapter.

2.1 Methodological workflow development

As outlined in the overarching aims (chapter 1, section 1.5.1), the development of a reliable and replicable workflow was essential to enable direct comparisons of the spatiotemporal molecular alterations occurring in synaptic isolates derived from varied species. The methodological model was established in years 1 and 2 and utilises a combinatorial approach, encompassing quantitative proteomics, *in silico* analyses and molecular genetics. The workflow was designed to be applicable to any spatiotemporal analysis, utilising any tissues, species or disease model of choice, to promote consistency between “-omic” experiments. For more information please refer to appendix 1 where my recommendations for experimental design and implementation of such workflows is documented in a published manuscript (*Expert Review of Proteomics*, 2016).

2.2 Ethics

In compliance with the 3Rs, no animals were bred specifically for this project. All tissue samples used in this thesis were derived from existing archived brains or harvested alongside other ongoing experiments. The University of Edinburgh internal ethics committee approved all animal experiments.

2.3 Human Patient Samples

Human patient samples were obtained from the Edinburgh Brain Bank in collaboration with Prof. Colin Smith. All tissues were classified as “controls” due to the absence of gross pathological hallmarks and neurological disease. Use of human tissue for post-mortem studies was reviewed and approved by the Edinburgh Brain Bank ethics committee. The Edinburgh Brain Bank is a Medical Research Council funded facility with research ethics committee approval (11/ES/0022).
2.4 Synaptosomal Preparations

Brain tissue samples were homogenised in an ice-cold isotonic sucrose solution (0.32 M sucrose, 1 mM EDTA, 5 mM Tris-HCl, pH 7.4). Homogenates were centrifuged in a fixed-angle rotor at 900 g for 10 min and the supernatant (S1) was collected. The pellet (P1) was resuspended in sucrose solution and centrifuged again at 900 g for 10 min. Supernatants were combined and centrifuged in a fixed angle rotor at 20,000 g for 15 min. The remaining pellets (P2) contained the synaptosomes. Such synaptosomal isolates are predominantly pre-synaptic in terms of content and demonstrate adequate purity for our experimental needs as previously demonstrated (156-158).

2.5 Mitochondrial Preparations

For a comprehensive overview of the methodology employed, see Lai et al, 1977 (63) and Fig. 12. This traditional approach was adopted due to the methodological technique resulting in samples with demonstrated organelle purity and conserved biochemical activity. All procedures were carried out at 4°C. Forebrains were homogenized by hand in isolation medium (0.32M sucrose, 1mM K-EDTA, 10mM, Tris HCl) to give a 1:10 homogenate. The homogenates were centrifuged at 1,300g for 3 minutes and the resulting pellets were manually resuspended in 15ml isolation medium before recentrifugation using the same parameters. Supernatants were pooled and centrifuged at 17,000g for 10 minutes to give the crude mitochondrial pellet (CM). The CM pellet was resuspended in 15ml isolation medium and 5ml of the suspension was layered into 3 tubes containing 7ml of 7.5% Ficoll-sucrose medium (7% Ficoll, 0.32M sucrose, 50µM K-EDTA, 10mM Tris HCl) on top of 7ml of 10% Ficoll-sucrose medium (10% Ficoll, 0.32M sucrose, 50µM K-EDTA, 10mM Tris HCl) and centrifuged at 99,000g for 30 minutes on a swing-out Beckman ultracentrifuge. This resulted in a myelin (My) fraction banded at the top, a synaptosomal (Syn) fraction at the second interphase and a ‘free’ (non-synaptic) mitochondrial pellet (M) at the bottom. The myelin fraction was aspirated and the synaptic fraction collected, without disturbing the pellet. This was then diluted in 3x isolation medium and centrifuged at 18,500g for 10 minutes. The non-synaptic
mitochondrial pellet was frozen immediately on dry ice before storage at -80°C. The resulting synaptosomal pellet was lysed and resuspended in 30ml 6mM Tris-HCl (pH 8.1) and centrifuged at 11,800g for 10 minutes. The supernatant was removed and the pellet was again resuspended in 10ml of 6mM Tris-HCl (pH 8.1) and recentrifuged at 8,300g for 10 minutes. The supernatant was discarded and the pellet resuspended in 10ml of 3% Ficoll medium (3% Ficoll, 0.12M mannitol, 30mM sucrose, 25µM K-EDTA, 5mM Tris-HCl). One third of this suspension was layered into 3 tubes containing 5ml 4.5% Ficoll medium (4.5% Ficoll, 0.24M mannitol, 60mM sucrose, 50µM K-EDTA, 10mM Tris-HCl) on top of 10ml 6% Ficoll (6% Ficoll, 0.24M mannitol, 60mM sucrose, 50µM K-EDTA, 10mM Tris-HCl) and centrifuged at 11,300g for 30 minutes in a swing-out Beckman ultracentrifuge. After centrifugation, the top band was removed and the intermediate band decanted and diluted with an equal volume of isolation medium. This lysate was then centrifuged at 17,000g for 10 minutes providing synaptosomally derived mitochondria (SM population) and an SM2 fraction (pellet). The SM and SM2 fractions were pooled to give a greater yield for proteomic experiments and validation, frozen on dry ice and stored at -80°C.
Figure 12: Method of mitochondrial neuronal subpopulation isolation from cortical tissue. Image adapted from Lai et al. Abbreviations: Syn – synaptosomal, M – non-synaptic, SM – ‘light’ fraction, SM2 – ‘heavy’ fraction.

2.6 Protein Concentration Assay

Samples were homogenized in label-free or RIPA buffer + 1% protease cocktail inhibitor (Thermo Scientific). After homogenisation, samples were centrifuged at 20,000g for 20 minutes at 4°C. The supernatant containing the solubilised protein was removed and pellets discarded. Protein concentration of samples was determined using a Pierce Micro BCA assay kit according to the manufacturers instructions.

2.7 Label-free Proteomics

All mass spectrometry analyses were performed by FingerPrints proteomic facility at the University of Dundee unless otherwise specified. I was afforded the opportunity
to implement the following methodology during a 7-day training course alongside Amy Tavendale and Douglas Lamont at the facility during my PhD studies. Samples were extracted in SDT lysis buffer containing 100 mM Tris-HCl (pH 7.6), 4% (W/V) Sodium dodecyl sulfate (VWR) and 0.1 M d/l-dithiothreitol (Sigma). For efficient protein extraction, lysates were freeze–thawed and homogenized in SDT buffer several times. Protein concentration was then determined using a micro BCA assay (see section 2.5).

Aliquots of each preparation were processed through FASP (filter-aided sample preparation) prior to a double digestion with trypsin (Roche, sequencing grade), initially for 4 hours, then overnight at 30 °C. Trypsin-digested peptides were separated using an Ultimate 3000 RSLC (Thermo Scientific) nanoflow LC system. Using an ESI Easy Spray source at 50 °C, technical replicates of each sample were loaded with a constant flow onto an Acclaim PepMap100 nanoViper C18 trap column (ThermoScientific). After trap enrichment, peptides were eluted onto an Acclaim PepMap RSLC nanoViper, C18 column (ThermoScientific). The HPLC system was coupled to a linear ion trap Orbitrap hybrid mass spectrometer (LTQ-Orbitrap Velos Pro, Thermo Scientific) via a nanoelectrospray ion source (Thermo Scientific). Full-scan MS survey spectra in profile mode were acquired in the Orbitrap. Data were acquired using the Xcalibur software.

All further analyses were performed by Laura Graham at the Roslin Institute. Raw proteomic data were imported into Progenesis for characterization and analysis of relative ion abundance. 2D representations of MS/MS output were created for each sample and these were aligned to determine similar features. Following alignment, data was filtered by retention time to correct for elution variability. The runs were grouped according to the experimental paradigm and Statistical P values were automatically generated in Progenesis software through a one-way ANOVA on the ArcSinh transform of the normalized data.

Peptides were filtered by the following criteria: power <0.8, fold change >1.2, p>0.05 and the remaining data were exported from Progenesis for identification of
individual peptide sequences using the Mascot Search Engine (V2.3.2). Identified proteins were re-imported into Progenesis for further processing. Proteins were subject to stringent filtering parameters to eliminate those which had <2 unique peptides, <1.2 fold change and p>0.05 to obtain the proteins which demonstrated the largest significant variation in expression between experimental conditions.

2.8 Biolayout Express\textsuperscript{3D}
Proteomic data was dissected using the complex pattern recognition software, Biolayout Express\textsuperscript{3D} (159). The software allows visualization of molecular networks by applying Markov clustering algorithms to raw proteomic data (MCL 2.2). All graphs were clustered using a Pearson correlation value specific to the input data.

2.9 Ingenuity Pathway Analysis
Ingenuity Pathway Analysis (IPA) experiments were performed as previously described (157), with the interaction data limited as follows: direct and indirect interactions; experimentally observed data only; 35 molecules per network; 10 networks per dataset. Prediction activation scores (z-score) were calculated in IPA. The z-score is a statistical measure of the match between an expected relationship direction and the observed protein expression. Positive z-scores indicate activation (orange) and negative z-scores indicate inhibition (blue) (160).

2.10 Quantitative Fluorescent Western Blotting
Quantitative fluorescent western blotting was performed as previously described (161). Samples were diluted to provide desired protein concentration. 4-15µg protein was loaded per well into Nu-PAGE\textsuperscript{®} Novex\textsuperscript{®} 4-12% Bis Tris mini-gels (Life Technologies) and transferred to PVDF membranes using an iBlot\textsuperscript{®} and Invitrogen gel transfer stacks. Membranes were incubated in primary antibodies at 4°C and secondary antibodies at room temperature (concentrations according to manufacturers instructions) and washed in 1x PBS before imaging on Li-COR Odyssey infrared scanner (see Tables 1 & 2 for antibody details). Protein expression was quantified utilising Odyssey software (Li-COR Biosciences).
Table 1: Primary antibodies utilized for western blotting experiments.

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<tr>
<td>Telomerase</td>
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<td>Ogdh</td>
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<td>Citrate synthase</td>
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<td>Abcam</td>
<td>ab71598</td>
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Table 2: Secondary antibodies used for western blotting.

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<td>Donkey anti-rabbit IRDye 680</td>
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2.11 Drosophila Stocks

Drosophila husbandry, genetics and phenotypic assessment skills were initially acquired whilst hosted in the Pennetta laboratory for a period of 9 months at the University of Edinburgh. Outside advice on experimental paradigms was sought from Dr. Marc Freeman (University of Massachusetts/Oregon Health and Sciences University), Dr. Lukas Neukomm (University of Massachusetts), Dr. Mary Logan (Oregon Health and Sciences University) and Prof. Richard Ribchester (University of Edinburgh) where appropriate. All Drosophila stocks utilised are listed in Table 3. Stocks were obtained from the Vienna Drosophila Research Centre (VDRC), Austria; FlyORF, Switzerland and Bloomington Drosophila Stock Center (BDSC), University of Indiana, USA. Flies were raised on standard cornmeal food in a
temperature-controlled laboratory (average room temperature 22°C). Mammalian genetic homology to *Drosophila* ortholog lines was determined by input of genes into DIOPT (DRSC Integrative Ortholog Prediction Tool (162): see Table 3). The *elav-Gal4* driver strain was used for all experiments described (see Table 4). Crosses were maintained at 22°C for 24 hours before removal of adults and embryos were incubated at 30°C to increase levels and activity of the *Gal4* proteins.

**Table 3: *Drosophila* lines utilized throughout the project.** DIOPT score indicates mammalian genetic homology to ortholog lines. Higher scores denote increased candidate homology. *Maximum score of 11.*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Source</th>
<th>Annotation symbol</th>
<th><em>Drosophila</em> stock ID</th>
<th>DIOPT score</th>
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<tbody>
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<td>VDRC</td>
<td>CG3192</td>
<td>v30413</td>
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<tr>
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<td>v9180</td>
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<td>v36166</td>
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**Table 4: *Drosophila* driver and control lines.**

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<th>Source</th>
<th>Description</th>
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<td>Canton-S</td>
<td>BDSC</td>
<td>Wild-type</td>
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<tr>
<td><em>elav</em>&lt;sup&gt;C155&lt;/sup&gt;-<em>Gal4</em></td>
<td>Dr. Giuseppa Pennetta</td>
<td>Nervous system expression driver</td>
</tr>
</tbody>
</table>
2.12 Immunohistochemistry

Wandering third instar larva were selected and dissected in PBS (n=8). The dissected larval neuromuscular junctions (NMJs) were fixed in Bouin’s fixative (15:5:1 picric acid, 37% formaldehyde and acetic acid) for 10 minutes and washed thoroughly in PBT (PBS + 0.1% TritonX-100). Preparations were blocked in PBT + 10% normal goat serum for 2 hours then incubated in primary antibody overnight at 4°C. NMJs were again washed extensively in PBT and incubated in secondary antibody at room temperature for 2 hours (see Tables 5 & 6 for antibody details). Samples were mounted on microscope slides using Vectashield mounting medium (Vector Laboratories) and imaged on a Zeiss confocal microscope. All quantification was performed in ImageJ using a standardised protocol for NMJ analyses (163).

Table 5: Primary antibodies employed for *Drosophila* immunohistochemistry experiments.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Source</th>
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<td>Bruchpilot</td>
<td>Developmental Studies Hybridoma Bank</td>
<td>nc82-s</td>
</tr>
</tbody>
</table>

Table 6: Secondary antibodies utilised for *Drosophila* immunohistochemistry experiments.

<table>
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<th>Secondary antibody</th>
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<td>Jackson ImmunoResearch</td>
<td>111-545-003</td>
</tr>
<tr>
<td>Cy3-AffiniPure Goat Anti-Mouse IgG</td>
<td>Jackson ImmunoResearch</td>
<td>115-165-146</td>
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</table>
2.13 Statistical Analyses
Data were collected in Microsoft Excel and statistical tests were performed in GraphPad Prism 6 software. For all analyses p<0.05 was considered significant. Statistical tests used are detailed in the results or figure legends where appropriate.
Chapter 3. Proteomic profiling of neuronal mitochondria reveals modulators of the synaptic architecture

I confirm that I have authored the following work and that this chapter is currently under review at Molecular Neurodegeneration (August 2017).

3.1 Introduction

Ageing is a fundamental risk factor for the development of a large range of neurodegenerative diseases, which are characterized by the selective death of neuronal subpopulations (52, 164). Neurons are highly polarized cells consisting of 3 distinct functional domains: the cell body, axon and the synapse. Previously, it was believed that the clinical phenotypes of neurodegenerative diseases were caused by the loss of entire neurons (165), however it has recently become apparent that these neuronal sub-compartments can degenerate independently of one another (166, 167), with synapses being particularly vulnerable to a broad range of stimuli. Whilst the properties of the potential differential degenerative mechanisms remain largely unknown, numerous themes have consistently appeared in the literature, suggesting that proteins regulating the ubiquitin-proteasome system (14, 168, 169), oxidative stress (62, 66, 170, 171) and mitochondria (14, 52, 59, 60, 66, 171, 172) may all play a role in regulating the stability of the synaptic compartment.

Synaptic compartments constantly demand ATP to maintain ionic gradients and neurotransmission events (173). In addition synapses also demonstrate a substantial need for calcium buffering machinery (172). Accordingly, sub-populations of mitochondria are enriched pre- and post-synaptically (14, 52, 66). Such synaptic mitochondria are reportedly distinguishable from non-synaptic mitochondria, displaying unique enzymatic (63), calcium buffering (64, 65) and antioxidant properties (66). However, if and how these distinctive subpopulations of synaptic mitochondria influence the vulnerability of synaptic compartments remains largely
unknown.

In an attempt to address this, we have used label-free proteomics to characterise the proteomes of synaptic and non-synaptic mitochondria following established biochemical isolation methods (63). By utilizing this methodology, we have generated proteomic profiles that reveal consistent molecular fingerprints for synaptic and non-synaptic mitochondria. Quantitative fluorescent western blotting was used to confirm these proteomic and fractionation data in a range of species, including rat and ovine tissues. These results were consistent with the existence of distinct mitochondrial sub-populations containing patterns of relative protein abundances that are conserved between different mammalian species. To determine if the molecular differences between these mitochondrial subpopulations may be capable of influencing the vulnerability of synapses, we manipulated the expression of mitochondrial candidates in vivo to assess synaptic stability in the Drosophila neuromuscular system. Our data demonstrate that selective knock-down of intrinsic mitochondrial proteins identified in this manner have the potential to alter synaptic morphology and the area of the presynaptic active zone in vivo. Thus, changes in mitochondrial protein expression may contribute to increased synaptic vulnerability and dysfunctional neurotransmission in early molecular pathological processes during ageing and/or disease.
3.2 Methods

3.2.1 Ethics
In compliance with the 3Rs, no animals were bred specifically for this project. Where possible all tissue samples used in this current study were derived from existing archived brains or harvested alongside other ongoing experiments. All animal experiments were approved by the University of Edinburgh Animal Welfare and Ethical Review Body and performed in accordance with the UK Animal (Scientific Procedures) Act, 1986. Use of human tissue for post-mortem studies was reviewed and approved by the Edinburgh Brain Bank ethics committee. The Edinburgh Brain Bank is a Medical Research Council funded facility with research ethics committee approval (11/ES/0022).

3.2.2 Rats
Nine male wild-type Sprague Dawley rats aged ca. 24 weeks (actual range 168-171 days) were used. Rats were group housed (3-4 per cage) in a SPF facility in open-top cages and maintained on a 12-12h light-dark cycle (lights on at 07:00h), under controlled temperature (22±2°C) and humidity (55±5%) with free access to drinking water and standard 14% protein rodent diet (Harlan Teklad). Rats were killed by conscious decapitation and brains were immediately excised. Brain stem and cerebellum were removed and discarded. Fresh forebrains were weighed and pooled for homogenization before mitochondrial preparations.

3.2.3 Sheep
Three Scottish blackface female sheep aged 5 years were utilized. Sheep were sacrificed at the Farm Animal Teaching Hospital, Royal (Dick) School of Veterinary Studies, University of Edinburgh by anaesthetisation and exsanguination. Brains were excised, bisected at the sagittal midline and the brain stem and cerebellum were removed and discarded. Tissues were submerged in ice cold high magnesium artificial cerebral spinal fluid (NaCl 125mM; NaHCO₃ 26mM; glucose 25mM; KCl 2.5mM; NaH₂PO₄(2H₂O) 1.25mM; CaCl₂ 1mM; MgCl₂ 4mM) to maintain brain
structural integrity before cortical mitochondrial preparations.

3.2.4 Mitochondrial preparations
For a comprehensive methodological outline see chapter 2, section 2.5 and Lai et al, 1977 (63). Synaptic and non-synaptic mitochondrial populations were isolated from the rat and sheep tissues described in sections 3.2.1 and 3.2.2. Protein concentrations of each sample was determine using the methods outlines in chapter 2, section 2.6.

3.2.5 Label-free proteomics
Synaptic and non-synaptic mitochondrial preparations were extracted in SDT lysis buffer containing 100 mM Tris-HCl (pH 7.6), 4% (W/V) sodium dodecyl sulfate (VWR) and 0.1 M d/l-dithiothreitol (Sigma). For efficient protein extraction, lysates were freeze–thawed and homogenized in SDT buffer several times. Protein concentration was then determined using BCA assay.

The following mass spectrometry analyses were performed by the FingerPrints proteomic facility at the University of Dundee. Aliquots (2mg) of each mitochondrial preparation were processed through FASP (filter-aided sample preparation) involving buffer exchange to 8 M urea and alkylation with 50 mM iodoacetamide prior to a double digestion with trypsin (Roche, sequencing grade), initially overnight, then for an additional 5 h at 30°C. Resulting peptides were desalted then separated using an Ultimate 3000 RSLC (Thermo Scientific) nanoflow LC system. Using an ESI Easy Spray source at 50 °C, technical replicates (3 × 0.75 μg) of each sample were loaded with a constant flow of 5 μL/min onto an Acclaim PepMap100 nanoViper C18 trap column (100 μm inner diameter, 2 cm length; Thermo Scientific). After trap enrichment for 3 min, peptides were eluted onto an Acclaim PepMap RSLC nanoViper, C18 column (75 μm, 50 cm; Thermo Scientific) with a linear gradient of 2–40% solvent B (80% acetonitrile with 0.08% formic acid) over 90 min with a constant flow of 300 nL/min. The HPLC system was coupled to a linear ion trap Orbitrap hybrid mass spectrometer (LTQ-Orbitrap Velos Pro, Thermo Scientific) via a nanoelectrospray ion source (Thermo Scientific). The spray voltage was set to 1.6 kV, and the temperature of the heated capillary was set to 250 °C. Full-
scan MS survey spectra ($m/z$ 335–1800) in profile mode were acquired in the Orbitrap with a resolution of 60,000 after accumulation of 1,000,000 ions. The 15 most intense peptide ions from the preview scan in the Orbitrap were fragmented by collision-induced dissociation (normalized collision energy, 35%; activation $Q$, 0.250; and activation time, 10 ms) in the LTQ after the accumulation of 10,000 ions. Dynamic exclusion parameters were set as follows: repeat count, 1; repeat duration, 30 s; exclusion list size, 500; exclusion duration, 45 s; exclusion mass width, plus/minus 10 ppm (relative to reference mass). Maximal filling times were 1,000 ms for the full scans and 150 ms for the MS/MS scans. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged species were rejected. The lock mass (445.120 024), option was enabled for survey scans to improve mass accuracy. Data were acquired using the Xcalibur software (for all raw data see PRIDE project accession: PXD005537).

The following experiments were performed by Laura Graham at the Roslin Institute. Raw proteomic data were imported into Progenesis for characterization and analysis of relative ion abundance. 2D representations of MS/MS output were created for each sample and these were aligned to determine similar features (average alignment score $>$90%). Following alignment, data was filtered by retention time with features detected below 5 minutes and above 110 minutes discarded to correct for elution variability and peptides with charge state between 2 and 5 only included in the search. The runs were grouped according to subcellular localization (synaptic and non-synaptic) and Statistical $P$ values were automatically generated in Progenesis software through a 1 way ANOVA on the ArcSinh transform of the normalized data. Peptides were filtered by the following criteria: power $<$0.8, fold change $>$2, $p<0.05$ and the remaining data were exported from Progenesis for identification of individual peptide sequences using the Uniprot Swall subspecies rattus norvegicus via Mascot Search Engine (V2.3.2). Enzyme specificity was set to that of trypsin, allowing for cleavage N-terminal to proline residues. Other parameters used were as follows. (i) Variable modifications: methionine oxidation, methionine dioxidation, protein N-acetylation, gln $\rightarrow$ pyro-glu. (ii) Fixed modifications: cysteine carbamidomethylation. (iii) MS/MS tolerance: FTMS- 10 ppm, ITMS- 0.6 Da. (iv)
Maximum missed cleavages: 2. (vi) False Discovery Rate: 1%. A cutoff score of >29 was used based on Mascot probability threshold of 0.05 that the observed hit is a random event. As an indication of identification certainty, the false discovery rate for peptide matches above identity threshold was set at 1%. Identified proteins were re-imported into Progenesis for further processing. Proteins were subject to stringent filtering parameters to eliminate those which had <2 unique peptides, <2-fold change between subpopulations and p>0.05 to obtain the proteins which demonstrated the largest significant variation in expression between synaptic and non-synaptic mitochondria.

3.2.6 Quantitative Fluorescent Western Blotting
For a comprehensive methodological outline see chapter 2, section 2.10 and Eaton et al., 2013 (161). Western blotting was performed on synaptic and non-synaptic mitochondrial isolates derived from both the rat and sheep cortical tissues. All antibodies details are listed in Tables 1 & 2.

3.2.7 Transmission Electron Microscopy
Control mixed sex human patient brain samples (n=4) were prepared for electron microscopy as previously outlined in detail (174). Briefly, fresh post-mortem samples, stored in 0.1 M PB were trimmed into small cortical blocks and fixed in 4 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M PB for 48 h. Mouse brain samples were prepared as follows: anesthetized WT male C57BL/6J mice ((n=4) intraperitoneal injection of Ketanest (100 mg/kg) and Rompun (5 mg/kg)) were killed by perfusion fixation with 0.1M phosphate buffer containing 4% paraformaldehyde and 2.5% glutaraldehyde before removing the brain and immersing it in fixative for a further 12 h. Following fixation, both human and mouse brain samples were washed in 0.1 m phosphate buffer before cutting free floating 70-µm-thick coronal sections on a Vibratome. Sections were postfixed in 1% osmium tetroxide in 0.1 m phosphate buffer for 45 min. Following dehydration through an ascending series of ethanol solutions and propylene oxide, all sections were embedded on glass slides in Durcupan resin. Regions of interest were glued onto a resin block for sectioning. Ultrathin sections (60–70 nm) were cut and
collected on Formvar-coated grids (Agar Scientific), stained with uranyl acetate and lead citrate in an LKB “Ultrostainer”, and then quantitatively assessed in a Philips CM12 transmission electron microscope. Negatives taken in the microscope were scanned into an Apple Macintosh G5 computer using an Epson 4870 Photo flat-bed scanner at 600dpi and subsequently processed using Adobe Photoshop. Images were analysed using ImageJ. Prof. Tom Gillingwater performed the electron microscopy experiments and provided control human patient and WT mouse cortical images for analysis.

3.2.8 Ingenuity Pathway Analysis
Please refer to chapter 2, section 2.9 for a detailed overview of the methodological parameters employed for pathway analysis.

3.2.9 Drosophila Stocks
*Drosophila* stocks were obtained from the VDRC (IDs: 21707, 47615, 101336, 30413) and Bloomington *Drosophila* stock center (Canton-S). For more information on the stocks utilised and homology of the *Drosophila* ortholog with regards to the mammalian gene please refer to Tables 3 & 4. Off-target effects of candidate lines were assessed using E-RNAi software (175). Candidate lines exhibited no off-target effects based on genetic mapping data and all displayed 97.21-100% efficiency for targeting the intended gene (Ndufb8 = 97.75%; mitofilin = 97.21%; Vdac1 = 100%; Aldh = 100%). The *Drosophila* experiments described in this chapter were performed in conjunction with Dr. Giuseppa Pennetta.

3.2.10 Statistical Analysis
Data were collected in Microsoft Excel and statistical tests were performed in GraphPad Prism 6 software. For all analyses p<0.05 was considered statistically significant. Statistical tests used are detailed in the results or figure legends where appropriate.
3.3 Results

3.3.1 Mitochondrial subcellular localisation dictates organelle morphology in rodents and humans

Enzymatic activity differences between neuronal mitochondrial sub-populations were first comprehensively described in the 1970s (63). However, the molecular underpinnings of these biochemical differences and their morphological or physiological consequences were never elucidated. We therefore began by investigating whether mitochondria residing at the pre-synaptic terminal were morphologically different from those in non-synaptic compartments. It has previously been suggested that the size of mitochondria may provide indications of biochemical processes as well as neuronal integrity and survival. Numerous studies have begun to describe mitochondrial morphology in the cell body (66, 176-178) but few demonstrate characterisation of those organelles in the axon and synapse – the compartments that show heightened vulnerability to degenerative insult. To establish whether size differences existed between synaptic and non-synaptic mitochondria, we examined transmission electron microscopy images from human and mouse cortical tissue. Mitochondria were classified as ‘synaptic’ if they were <1µm from the post-synaptic density and vesicles were clearly present within the terminal. Mitochondria outside of the boutons were classed as non-synaptic; anything ambiguous was excluded from the analysis. The results indicate that in both mouse and human cortex, non-synaptic mitochondria appear significantly larger than the organelles present within synaptic terminals ((Fig. 13) mouse: p<0.0001; human: p=0.0099, respectively). Non-synaptic populations in both species demonstrated elongation versus that of synaptic mitochondria, which displayed a spherical morphology (data not shown). Although these morphological differences between mitochondrial sub-populations may solely reflect adaptations to the particular size and structure of cellular compartments, we hypothesised that biochemical adaptations would also likely exist due to the dynamic interplay between protein expression and organelle configuration (see Picard et al, 2013 for a comprehensive review on mitochondrial morphology and function (54)).
Figure 13: Synaptic and non-synaptic mitochondria are morphologically distinct in both rodent and human brain tissue. A&B. Example EM images of WT mouse and human cortical tissue displaying synaptic and non-synaptic mitochondria (S = synaptic, NS = non-synaptic). C&D. Scatterplots representing WT mouse (C.) and control human patient (D.) cortical synaptic and non-synaptic mitochondrial areas (µm²). C. synaptic n=391, non-synaptic n=1113 from 4 mice; D. synaptic n=177, non-synaptic n=812 from 4 patients. A data point of distinct shape represents each individual animal or patient. Non-synaptic mitochondria appear to be significantly larger than synaptic mitochondria in both mouse and human cortical tissue. Student’s t-test with Welch correction for unequal sample size: ****p<0.0001, **p<0.01). Scale bar = 0.5µm.
3.3.2 Label-free proteomics reveals significant mitochondrial heterogeneity

To identify molecular differences between mitochondria derived from synaptic and non-synaptic neuronal compartments, we performed label-free proteomics on mitochondrial-enriched fractions isolated from rat forebrain. Quantitative label-free proteomic analyses identified >1500 proteins associated with both synaptic and non-synaptic mitochondria, revealing dynamic variations in protein expression dependent upon subcellular localisation (Fig. 14A&B). Strikingly, over 400 proteins were altered by greater than 2-fold between mitochondrial subpopulations (Fig. 14C), demonstrating significant compartment-dependent biochemical adaptations.
Relative purity of mitochondrial isolates was assessed using bioinformatics enrichment analyses (Table 7) and quantitative fluorescent western blotting (QFWB) on ovine forebrain. The mitochondrial yield obtained from rat forebrain was not sufficient for validation purposes (see methods). The mitochondrial markers VDAC1 and COXIV indicated significant enrichment in synaptic and non-synaptic mitochondrial fractions versus whole brain lysate suggesting purification of the mitochondria from their respective subcellular compartments (Fig. 15A). In addition to demonstrating purification of mitochondrial subpopulations, we again employed QFWB to determine the veracity of the proteomic data. We observed corresponding protein expression trends in each tissue preparation, as indicated by the proteomics, for multiple proteins (Fig. 15C-E). Taken together, these results indicate the relative purity of the mitochondrial isolates and suggest the proteomic data is representative of the molecular alterations occurring in the tissue samples. Furthermore, the data indicate that the observed heterogeneity in mitochondrial protein expression may be
conserved between mammalian species.

Figure 15: Purification of distinct mitochondrial subpopulations and validation of proteomic data. A. Quantitative fluorescent western blots demonstrating relative abundance of the mitochondrial markers COXIV and VDAC1 and the nuclear marker telomerase in whole brain lysate and mitochondrial samples. Mitochondrial samples display little nuclear contamination and enrichment of COXIV and VDAC1. B. Quantification of the beta-tubulin loading control signal. C. Left bar chart displays the proteomic average normalised expression values of proteins in synaptic and non-synaptic mitochondria. Right bar chart demonstrates sample protein expression quantified by fluorescent western blots. Proteomic and sample expression of all proteins (HIBCH, OGDH and citrate synthase) follow the same trend thereby providing validation of the proteomic data. Statistical analysis was performed using an unpaired two-tailed Student’s t-test, n=3.

Table 7: DAVID 6.7 (NIAID/NIH) enrichment analysis of synaptic and non-synaptic mitochondrial samples. Table comprises the top gene ontology (GO) terms associated with 1511 proteins identified in synaptic and non-synaptic mitochondrial samples. The analysis
displays predominantly mitochondrial components, suggesting the samples are enriched for mitochondria.

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<th>Enrichment Score</th>
<th>Count</th>
<th>P_Value</th>
<th>Fold Change</th>
<th>Benjamini</th>
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<td>4.70E+00</td>
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### 3.3.3 Expression profiling highlights bioenergetic alterations between synaptic and non-synaptic mitochondrial proteomes

Having confirmed the veracity of the proteomic data, we next sought to further dissect the proteomic data using Biolayout Express3D (159). The software applies unbiased Markov clustering algorithms to the input data and groups proteins displaying similar expression trends. This allows visualisation of spatial profiles promoting the identification of physiological cascades altered within the dataset. The nodes, shown as spheres with identical dimensions, signify individual proteins and the edges, represented by connecting lines, are indicative of the correlation of protein expression within the data. Graphs were constructed in 3D space utilizing the 1511 identified proteins from the mitochondrial proteomic analysis (see Fig. 14B&C), generating 10 protein clusters. In agreement with the principal component analysis correlation graphs (Fig. 14D&E), network clustering of the data displayed similar trends with regards to fragmentation of the graph into two localisation-dependent networks (Fig. 16). The fragmentation of the dataset into 2 distinct groups suggests that significant heterogeneity in protein expression exists between the mitochondrial populations derived from discrete neuronal sub-compartments. Indeed, with further examination we exhibited that proteins with increased abundance in synaptic
mitochondria cluster into the distinct network on the left of the graph whereas those with increased abundance in non-synaptic mitochondria are observed in the right network (Fig. 16). To identify the functional cascades associated that appear to demonstrate differential expression between the two mitochondrial subpopulations, the top 3 protein clusters were entered into Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 to determine the basic functions of the clustered proteins (Fig. 16). Interestingly, the proteins that exhibit upregulation in synaptic mitochondria (clusters 1 and 2) are associated with the inner membrane bioenergetic complexes, which suggests that distinct synaptic and non-synaptic mitochondrial protein expression may reflect altered local requirements for energetic expenditure. Interestingly, these data correlate with the original biochemical study by Lai et al (63), which reported variations in mitochondrial enzymatic activities dependent upon the subcellular localisation of the organelles. Here, we can account for approximately 35% of those reported enzymatic activity alterations, which are likely due to the differing abundance of particular enzymes in the discrete sub-pools of mitochondria.

![Figure 16: Expression profile clustering identifies alterations associated with bioenergetic control.](image)

Nodes (spheres) represent individual proteins and edges (lines) reflect the strength of correlation of expression between proteins. The schematic displays 2 distinct protein networks – proteins displaying increased abundance in synaptic mitochondria on the left and those exhibiting enhanced expression in non-synaptic mitochondria on the right. Coloured nodes represent protein clusters based on expression profile and each cluster
3.3.4 Pathway analysis highlights mitochondrial complex I expression differences as potentially conserved regulators in a range of neurodegenerative conditions.

In order to determine how differential protein expression in mitochondrial subpopulations may synergistically regulate downstream molecular pathways and cellular processes, we performed an in silico analysis using Ingenuity Pathway Analysis (IPA) software. The software promotes the identification of statistically significant functional groups of proteins, based on known protein interactions and biological functions reported in the published literature (157). Functional networks generated by IPA software are statistically ranked according to a score calculated via a right-tailed Fischer's exact test, taking into account the number of original input proteins and the size of the resulting network. With input of the 411 differentially expressed proteins from the mitochondrial proteomic data (>2-fold change), the analysis revealed that 154 (37%) candidates have previously been associated with neurological diseases in the published literature (Fig. 17A), suggesting that a multitude of these proteins may play a role in regulating synaptic stability.

Further examination into the molecular cascades that may be associated the pathogenesis of neurodegenerative diseases highlights that mitochondrial complex I is an upstream regulator of degenerative processes (Fig. 17B). The particular expression of proteins downstream of complex I in synaptic, but not non-synaptic, mitochondria have previously been implicated in numerous neurodegenerative diseases including Huntington’s disease, Parkinson’s disease and Lewy body dementia - all of which demonstrate synaptic perturbations early during pathogenesis (for a comprehensive review see (52)). These results suggest that modulation of the synaptic mitochondrial proteome may synergistically drive the demise of the synapse in age-related neuropathologies. In conjunction, assessment of the downstream physiological cascades modulated by synaptic mitochondrial protein expression
identified significant inhibition of synaptic transmission cascades (Fig 17C). The increased expression of alpha-synuclein, GAD1, SH3GL1, SH3GL2, S100B PPP3R1 and SYT1 in the non-synaptic mitochondrial proteome appears to indirectly inhibit synaptic transmission. Conversely, the enhanced expression of SNPH, PRNP, GNAI1 and GNAI2 in synaptic mitochondria inhibits neurotransmission cascades in the synaptic compartment. This suggests that heterogeneity in discrete mitochondrial proteomes and their associated downstream pathways has the propensity to promote functional alterations at the synaptic terminal.
Figure 17: Pathway analysis highlights mitochondrial alterations associated with
altered synaptic function and a broad range of neurodegenerative diseases, mediated by complex 1. A. 411 differentially expressed mitochondrial proteins are implicated in the literature as contributors to neurological disease, psychological disorders and skeletal and muscular disorders. B. Mitochondrial complex 1 is central to the network of interactions (highlighted in light blue) and features a number of intrinsic mitochondrial proteins and 2 protein families. Expression of the synaptic mitochondrial proteome is associated with perturbed synaptic structure and function. C. The significant differential expression of proteins in synaptic and non-synaptic compartments appears to inhibit downstream pathways associated with synaptic transmission. Solid lines indicate direct interactions; dashed lines represent indirect interactions; proteins highlighted in pink are those which are more abundant in synaptic mitochondria; proteins in green are those which are more abundant in non-synaptic mitochondria; dark blue represents predicted pathway inhibition. Shapes are indicative of protein function: diamond = enzyme; rectangle = GPCR; square = cytokine; trapezoid = transporter; circle = other.

3.3.5 Mitochondrial proteins regulate synaptic morphology in vivo

Although we had generated a compendium of the molecular differences present between synaptic and non-synaptic mitochondrial populations, it remained unclear if any of the identified proteins were capable of actively modulating synaptic stability in vivo. To assess this, we employed a molecular genetic approach using the Drosophila larval neuromuscular junction (NMJ) to screen individual proteins to examine their potential role in regulating synaptic morphology. The larval NMJ is an excellent model system to unravel the molecular mechanisms underpinning synaptic structure, function and plasticity (151). Fundamental mammalian biological and neurological pathways demonstrate conservation in Drosophila and recent evidence has indicated that up to 75% of human disease related genes exhibit a functional orthologue in the fruit fly (145). Larval NMJs harbour glutamatergic synaptic boutons with contiguous invaginated post-synaptic membranes (153, 154), which display significant homology to those found in the mammalian central nervous system. Neurotransmission between these connections has the propensity to invoke plastic modifications (151, 153, 155), providing physiologically relevant mechanistic insights into proteins regulating the structure and function of the synapse in
mammalian cognition (155).

In order to assess whether the expression of particular proteins in synaptic mitochondria may be regulating the morphology of the synapse, we utilised the *Drosophila UAS/Gal4* system, which allows tissue specific expression of a particular transgene with use of selected drivers. We selected candidates based on magnitude of change, mitochondrial localisation and availability of *Drosophila* orthologues (see Table 2). Interestingly, the short-listed candidates displayed increased protein expression in synaptic versus non-synaptic mitochondria. Thus, we aimed to selectively knock-down the expression of these mitochondrial proteins in third instar larva neurons for assessment of associated phenotypes. Pan-neural expression of the RNAi constructs (v30413; v47616; v10136; v21707) under control of the *elav-Gal4* driver resulted in viable larva from all crosses. To assess synaptic morphology and potential mitochondrial-dependent alterations in synaptic transmission, we examined the larval muscle 12/13 NMJ by immunohistochemistry with antibodies against the presynaptic marker horseradish peroxidase (HRP) and active zone marker bruchpilot (BRP). Here, we found that the selective RNAi-mediated knock-down of single candidates produced striking synaptic phenotypes at the NMJ (Fig. 18). NDUFB8, mitofilin, VDAC1 and ALDH demonstrated varied phenotypes at the NMJ with alterations in distinct synaptic parameters including bouton diameter and total bouton area (Fig. 18). The most severe phenotype was associated with selective knock-down of aldehyde dehydrogenase (ALDH), which promoted significant reductions (p<0.05) in synaptic bouton area with concomitant loss of distinguishable Ib and Is boutons (Fig. 18A). In conjunction, there was a significant decrease (p<0.0001) in synaptic bouton active zone staining (Fig. 19A) relative to control NMJs. Similarly, knock-down of neuronal NDUFB8 expression promoted aberrant NMJ morphology (Fig. 18A&B) with significant increases in bouton diameter. Despite this, the NMJs demonstrated a corresponding reduction in active zone area (p<0.0001) versus controls (Fig. 19A&C). Interestingly, we did not observe any obvious perturbations in axonal branching or morphology in our *elav-Gal4/RNAi* lines (Fig. 18F&G), suggesting that selective knock-down of these candidates promotes selective alterations in synaptic morphology, mediated by mitochondrial protein expression.
Significant reductions in the area of active zone staining in both the ALDH and NDUFB8 RNAi lines suggests that mitochondria may have a direct functional impact on vesicular release and bouton firing properties. Although the mechanistic pathways modulating these alterations remain elusive, regulation of the mitochondrial proteome appears to be significant mediator of synaptic-specific structural and biochemical properties.
Figure 18: Mitochondrial candidates modulate synaptic morphology in vivo. A. Representative image of WT muscle 12 NMJ in abdominal segment A3 (n=8) immunostained with anti-HRP. B-E. Top panel displays representative images of muscle 12 NMJ, hemisegment A3 bouton morphology with candidate knock-down. Middle panel demonstrates masks utilized for quantification of HRP staining in ImageJ. Lower panel
shows a section of axon. *Scale bar = 10um* for synaptic HRP and mask layers, *5um* for axons. **B.** elav-GAL4/Ndufb8RNAi; **C.** elav-GAL4/MitofilinRNAi; **D.** elav-GAL4/Vdac1RNAi; **E.** elav-GAL4/AldhRNAi. Arrows represent loss or alteration of boutons and/or fragmentation of dendritic branches. A variety of phenotypes exist. *All NMJs immunostained with anti-HRP and imaged at 63x (n=8).* **F.** Quantification of axon diameter in candidate lines versus control (Ndufb8 p=0.4095; Mitofilin p=0.169; Vdac1 p=0.1427; Aldh p=0.3138). **G.** Quantification of number of NMJ branches in candidate lines versus control (Ndufb8 p=0.1161; Mitofilin p=0.117; Vdac1 p=0.2722; Aldh p=0.0522). **H.** Quantification of the synaptic bouton diameter in candidate lines versus control (Ndufb8 p=0.0003; Mitofilin p=0.5759; Vdac1 p=0.0003; Aldh p=0.5291). **I.** Quantification of the total bouton area in candidate versus control lines (NDUFB8 p=0.4246; Mitofilin p=0.0078; Vdac1 p=0.6083; Aldh p=0.0337). *All quantification used students t-test; * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.*
Figure 19: Manipulation of the mitochondrial proteome modulates active zone expression. A. Representative images of WT, NDUFB8 and ALDH muscle 12 NMJs from abdominal segment A3 (n=8) immunostained with anti-HRP and anti-bruchpilot. Bottom panel displays ImageJ quantification mask utilized for analysis of HRP staining. B. Quantification of total bouton area in NDUFB8 and ALDH RNAi lines. C. Quantification of total NMJ active zone area in NDUFB8 and ALDH RNAi lines. All quantification used students t-test; * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. Scale bar = 10um.
3.4 Discussion

The current study has demonstrated novel insights into how a ‘top-down’ approach may be utilised for identifying individual proteins that may modulate the stability of synaptic compartments \textit{in vivo}. By using a range of techniques we have shown that neuronal mitochondria derived from synaptic and non-synaptic cellular compartments display significant morphological and biochemical differences. Electron microscopy analyses from mouse and human cortical tissue suggest that non-synaptic mitochondria are significantly larger than those found in the synaptic terminal. Alongside these morphological alterations, we have also demonstrated that upwards of 400 mitochondrial associated proteins identified from our label-free proteomic experiments display a $\geq 2$-fold expression difference between sub-populations. Using molecular genetic tools at the \textit{Drosophila} neuromuscular junction, we were able to manipulate the expression of 4 of the identified candidates that displayed increased abundance in synaptic mitochondria, resulting in selective alterations in synaptic, but not obvious axonal morphology \textit{in vivo}. Our data promote further understanding of the basic biology of the mitochondria and how these organelles may regulate the vulnerability of the synapse in a physiologically relevant model. In addition, the results presented may provide increased insight into how mitochondrial perturbations influence the synaptic alterations commonly occurring during ageing and disease.

Our initial proteomic screen revealed a remarkably large and heterogeneous list of candidates that significantly differed in expression dependent upon mitochondrial subcellular localisation. Despite this heterogeneity, upon further analysis of the data utilising \textit{in silico} tools, it became apparent that many of these proteins have previously been associated with perturbations in synaptic transmission. Experimental manipulation of 4 of these candidates produced varied synaptic phenotypes. The differing morphological phenotypes likely reflect the functional specificities, biological roles and downstream cascades associated with the proteins. NDUFB8, a mitochondrial complex I subunit, produced a relatively mild phenotype at the NMJ with morphological overgrowth but retention of branching patterns seen in controls. The preservation of numerous morphological characteristics despite NDUFB8
knock-down may reflect a compensatory mechanism occurring within the elaborate multi-subunit complex I. Conversely, reductions in ALDH expression resulted in the most severe phenotype, with significant alterations in synaptic bouton morphology and striking alterations within the NMJ architecture. This is likely due to its primary role in protecting mitochondria from endogenous aldehydes generated by lipid peroxidation (179). With reductions in ALDH, it is probable that an accumulation of noxious metabolites occurs, resulting in cytotoxicity. In support of our observations, a previous study from our laboratory (158) reported that Drosophila harbouring mutant ALDH displayed spontaneous degeneration of axons in the olfactory receptor neuron, however the presence of a synaptic phenotype was not morphologically assessed in that system. Furthermore, there have been several recent reports documenting a potential detoxification role for ALDH specifically in populations of dopaminergic neurons (180). Notably, sub-populations of dopaminergic cells from the brains of Parkinson’s disease patients demonstrated a significant reduction in ALDH (181), which may contribute to alterations in synaptic stability resulting in the activation of degenerative cascades.

Despite NDUFB8 and ALDH demonstrating varied synaptic bouton phenotypes, both candidates promoted a significant reduction in the active zone area. Mitochondrial morphology and function has been associated with alterations in the efficacy of the presynaptic active zone in numerous investigations, however the molecular mediators regulating such events remain elusive. Synaptic transmission is a bioenergetically demanding process requiring the persistent aerobic production of ATP and buffering of intracellular Ca\(^{2+}\) concentrations. Evidence indicates that mitochondria redistribute and bind to the active zone in response to synaptic transmission for homeostatic regulation at the synapse (182, 183), promoting the controlled exocytosis underpinning neurotransmission. Disruptions in the mitochondrial proteome may have the propensity to alter the functional organisation and stability of the active zone by reductions in calcium buffering, ATP availability and/or vesicular endocytosis. Although we directly manipulated the expression of NDUFB8 and ALDH, it is likely that the NMJ mitochondria harboured further disruptions to the proteome that were hierarchically driven by alterations in the candidates, which may have synergistically promoted aberrations at the active zone.
Although a conventional RNAi screen would require multiple RNAi and point mutations to be certain of the importance of specific candidates, we have presented several novel synaptic specific phenotypes with selective knock-down of single mitochondrial candidates. However, it is unlikely that the expression of these proteins solely regulate synaptic morphology and stability. Instead, it is probable that multiple cellular and molecular pathways, up-and downstream of the mitochondria, converge to modulate compartmental stability in mammalian systems. Despite this, protein expression within the mitochondria, as well as the subcellular localisation of the organelle, are clearly important variables that must be considered when characterising cascades negatively affecting synaptic morphology, transmission and physiology.

A next logical step in determining how the mitochondrial proteome may be contributing to synaptic vulnerability in vivo, is to perform an ageing study using the same tools and techniques presented in the current investigation. It has been well documented that mitochondria dynamically alter dependent on their environment (63-66), and we have provided further evidence for this hypothesis here. However, few studies have attempted to investigate how the synaptic mitochondrial proteome alters during ageing and how variations in protein expression may impact on the function and architecture of the synapse in a physiologically relevant model. An examination of such processes provides scope to potentially identify mitochondrial proteins that may be contributing to synaptic demise during normal healthy and pathological ageing. Furthermore, these data may indicate why such large disparities appear to exist with regards to the vulnerability of synaptic and neuronal populations with different pathogenic insults.

Although the use of label-free proteomics has provided a fairly comprehensive coverage of the mitochondrial proteome (this study yields the highest identification of mitochondrial proteins in a single in vivo analysis to date (1511)), for improved detection of low molecular weight proteins other techniques such as tandem mass tagging with high fractionation may be employed for future experiments. It is highly likely that numerous mitochondrial proteins that were not detected or removed from the data due to stringent filtering parameters, may influence synaptic stability in vivo.
Despite this, it is abundantly clear that proteomics and molecular genetics are powerful tools to identify candidates that may impact upon synaptic structure and function.

The current study has demonstrated novel insights into how a ‘top-down’ approach may be utilised for identifying novel candidates that may modulate the stability of synaptic compartments in vivo. Our investigation has established that the morphological and biochemical properties of synaptic and non-synaptic mitochondria differ significantly across a multitude of mammalian species. Using molecular genetic tools at the Drosophila neuromuscular junction, manipulation in the expression several candidates that displayed increased abundance in synaptic mitochondria, resulted in selective alterations in synaptic, but not axonal morphology in vivo. Thus changes in mitochondrial protein expression may contribute to increased synaptic vulnerability in early molecular pathological processes during ageing and/or disease.
Chapter 4. The ageing mitochondrial proteome and regulation of the synaptic milieu

4.1 Introduction

As alluded to in Chapter 3, mitochondria are incredibly dynamic and heterogeneous organelles that have the propensity to directly modulate the morphology of the synaptic architecture. Although mitochondria have long been considered a biochemical substrate significantly contributing to the dysfunction of neuronal compartments during advancing age (184), the mechanisms orchestrating such events and how these may impact upon cognitive function remain poorly understood. Traditional theories of age-dependent mitochondrial dysfunction, such as the free radical theory of ageing (92) - which suggests that mitochondrial reactive species cumulatively promote cellular damage during the lifespan - are now beginning to be revised due to novel experimental evidence languishing numerous facets of the model (184, 185). In recent years, a wealth of innovative studies has promoted the development of an alternative multi-factorial model of mitochondrial ageing suggesting that alterations in reactive species detoxification (186), bioenergetics (187), Ca$^{2+}$ buffering capacity (188), mtDNA integrity (189) and organelle dynamics (190) synergistically facilitate changes in neuronal receptivity during advancing age.

As previously discussed, synapses are particularly susceptible to the effects of advancing age, however the biochemical cascades governing this compartmental vulnerability remain elusive. Accordingly, recent investigations have begun to delineate how temporal alterations in mitochondrial subpopulations may be contributing to perturbations in the synaptic milieu and facilitating concomitant cognitive decline. Indeed, morphometric analyses of mitochondria using 3D electron microscopy techniques in young and aged rhesus macaques revealed significant age-dependent morphological alterations in organelles within cortical presynaptic terminals, which robustly correlates with cognitive capacity (190). Young animals exhibited stereotypical spherical and tubular mitochondria, whereas old animals appeared to harbour numerous “donut” shaped organelles, which are considered to be
indicative of mitochondrial dysfunction. Intriguingly, the number of “donut”
mitochondria directly correlated with the size of the presynaptic active zone and
number of synaptic vesicles, in addition to the severity of cognitive impairment,
suggesting that divergent mitochondrial morphologies may unequivocally modulate
synaptic transmission, plasticity and stability during advancing age (190, 191). In
agreement with this, investigations examining synaptic mitochondrial motility have
reported that the presence of mitochondria within the presynaptic compartment
dictates the strength of transmission (192). Retrograde trafficking of mitochondria
away from the presynaptic terminal mediates reductions in synaptic strength,
whereas the presence of the organelles in apposition to the active zone promotes
sustained neurotransmitter release and plastic modifications within the synaptic
terminal (192). Together, these data indicate that synaptic mitochondria play a
fundamental role in regulating a multitude of properties at the synapse (191) and
dynamic temporal modifications of this discrete population of organelles may
significantly impact upon the structure and function of the compartment, promoting
alterations in cognitive capacity.

The outlined studies documenting the dynamic intersection between mitochondrial
morphology and synaptic function during ageing are particularly compelling,
highlighting the influence of mitochondria on cognition. Molecular investigations
attempting to unravel the causal and/or concomitant temporal biochemical alterations
regulating this shift in synaptic mitochondrial morphometry however, demonstrate
significantly variable results, with reports describing conflicting changes in
important modulatory processes such as respiratory capacity (187, 193-196) and the
fission/fusion balance (194, 196, 197). Indeed, it is well established that the dynamic
regulation of mitochondrial morphology by fission and fusion events mediates
mtDNA integrity, respiration and free radical generation, and equilibration of these
processes appears critical during ageing (198, 199). Notably, numerous studies report
reductions in the expression of fission proteins in aged cells (200, 201), shifting the
fission/fusion equilibrium. Fission enables the segregation and sequestration of
damaged organelles via autophagy pathways, promoting the maintenance of a
healthy pool of functional mitochondria (60). Age-dependent decreases in proteins
regulating fission are associated with “giant” and “donut” dysfunctional
mitochondria (198), in addition to reduced numbers of organelles within synaptic terminals, which mitigate synaptic vesicle mobilisation during neurotransmission (60, 190). Reductions in fission events have also been directly associated with attenuated autophagy, suggesting that modifications in neuronal mitochondrial morphology may directly modulate proteostasis (201). Despite this, conflicting studies have indicated that age-dependent increases in mitochondrial fission may also be detrimental to the synapse. Investigations modelling numerous age-related neurodegenerative diseases in vitro report excessive fission-mediated mitochondrial fragmentation as an early pathogenic event (202), promoting organellar and cellular dysfunction and damage.

Of course, mitochondrial morphology and function are not mutually exclusive and a reciprocal effect exists upon physiological alteration of either parameter (203). Thus, investigations have begun to examine whether the respiratory capacities of synaptic and non-synaptic mitochondria alter with advancing age and identify how these changes may bolster compartmental vulnerability. Indeed, a recent proteomic study examining the temporal alterations occurring in the mouse synaptic mitochondrial proteome reported that increased age promoted the progressive downregulation of mitochondrial electron transport chain subunits, however, this appeared to have no functional impact on the bioenergetic capacity of isolated synaptic mitochondria (194). Despite this, there are now a number of studies disputing these results and investigations employing similar biochemical methodologies reveal that synaptic mitochondrial respiratory capacity is diminished with advancing age (187, 193, 204). Although, the reported respiratory data is not in agreement with the literature, the investigation also documented an accumulation of synaptic mitochondrial mtDNA damage, increases in reactive species and reductions in the expression of fission proteins (194), which appear to closely coincide with the studies highlighted above (186, 189, 200, 201). Although the adoption of proteomic methodologies to track the molecular spatiotemporal alterations occurring in synaptic mitochondria is an admirable approach, the authors did not include a non-synaptic mitochondrial population for proteomic comparison, thus preventing delineation of how subpopulation-specific biochemical alterations may be modulating compartmental vulnerability during ageing. It is imperative that future studies address the subcellular
divergence in temporal mitochondrial protein expression to enable identification of functional mediators that may be promoting perturbations in the synaptic compartment during advancing age.

Mitochondria unequivocally exhibit subcellular heterogeneity in morphology and protein expression (refer to Chapter 3) and studies are now beginning to demonstrate that the synaptic mitochondrial proteome may be dynamically altered during ageing (194). However, the presence of discrepancies regarding the temporal regulation of mitochondrial properties in the literature has hampered the identification of functional mitochondrial mediators that may be modulating synaptic stability and cognitive decline during advancing age. There is significant demand for well-executed, physiologically relevant studies that characterise the spatiotemporal molecular alterations occurring in both synaptic and non-synaptic mitochondria to enable the identification of biochemical cascades that may be promoting age-dependent vulnerability of the synaptic compartment.

In order to address this, here we employ an unbiased combinatorial approach, including quantitative proteomics coupled with in vivo phenotypic assessments, to temporally profile the biochemical alterations occurring in mitochondrial subpopulations throughout normal healthy ageing in the rodent cortex. We demonstrate that mitochondrial ageing is highly heterogeneous and subcellular localisation dictates the biochemical composition of discrete mitochondrial populations throughout the lifespan. Strikingly, we reveal that over 1000 proteins are temporally altered by greater than 20% in both synaptic and non-synaptic mitochondrial populations, demonstrating significant age-dependent modifications at the proteome level. Interestingly, aged synaptic mitochondria exhibit significant divergence in global protein expression, which may contribute to the enhanced vulnerability of cortical synapses at this particular age. Recapitulation of aged synaptic mitochondrial protein expression using molecular genetic tools in vivo revealed several novel functional mediators that have the propensity to significantly modulate multiple morphological parameters at the synapse. We suggest that selective alterations in the synaptic mitochondrial proteome may contribute to the
documented structural and functional perturbations occurring at synaptic terminals during advancing age.
4.2 Methods

4.2.1 Ethics
In compliance with the 3Rs, no animals were bred specifically for this project. All tissue samples used in the current study were derived from existing archived brains or harvested alongside other ongoing experiments. The University of Edinburgh internal ethics committee approved all animal experiments.

4.2.2 Animals
12 wild-type C57BL/6 mice of differing sexes and ages were utilized for the studies described. Animals were assigned to the young (mean age = 4 weeks), mid-age (mean age = 6 months) or old age (mean age = 24 months) group, with 4 animals per time point. Animals were killed by conscious decapitation and brains were immediately excised. Brain stem and cerebellum were removed and discarded and the remaining forebrains were frozen slowly in an insulated container at -80°C to preserve morphological integrity. Immediately before mitochondrial isolations, forebrains were rapidly thawed by immersion in a water bath at 37°C (for further information on slow-freeze/rapid thaw methodology please refer to Hardy et al, 1983 (205)). Samples were weighed and pooled by age group for homogenization and mitochondrial preparations.

4.2.3 Mitochondrial isolations
For a comprehensive methodological outline see chapter 2, section 2.5 and Lai et al, 1977 (63). Laura Graham performed all subcellular fractionation procedures. Synaptic and non-synaptic mitochondrial populations were isolated from the young, mid-age and old mouse tissues described in section 4.2.2. Protein concentrations of all samples were determined utilizing the methods outlined in section 2.6.

4.2.4 Label-free proteomics
Mass spectrometry was performed by Amy Tavendale, Samantha Kosto and Douglas Lamont at the University of Dundee. Synaptic and non-synaptic mitochondrial
preparations were extracted in SDT lysis buffer containing 100 mM Tris-HCl (pH 7.6), 4% (W/V) Sodium dodecyl sulfate (VWR) and 0.1 M d/l-dithiothreitol (Sigma). For efficient protein extraction, lysates were freeze–thawed and homogenized in SDT buffer several times. Protein concentration was then determined using BCA assay. Aliquots (200 µg) of each preparation were processed through FASP (filter-aided sample preparation) involving buffer exchange to 8 M urea and alkylation with 50 mM iodoacetamide prior to a double digestion with trypsin (Roche, sequencing grade), initially for 4 h, then overnight at 30 °C. Trypsin-digested peptides were separated using an Ultimate 3000 RSLC (Thermo Scientific) nanoflow LC system with the column oven set to 35 °C. Technical replicates (3 × ~1 µg) of each sample were loaded at a constant flow of 5 µL/min onto a trapping cartridge (PepMap100, C18, 5µm, 100Å 0.3 x 5 mm; (Thermo Scientific, San Jose, CA) using 2% Acetonitrile, 0.1 % formic acid. After trap enrichment, peptides were separated on a peptide CSH, 1.7µm, 130Å, 75µm x 250mm C18 column (Waters Corp, Milford, MA) with the following gradient: t=0 min, 2 % B; t=6, 2 % B; t=20, 8 % B; t=110, 24 % B; t=135, 37 % B where solvent A is water with 0.1 % formic acid and solvent B is 80% acetonitrile with 0.1% formic acid, with a constant flow of 260 nL/min. The HPLC system was coupled to a linear ion trap Orbitrap hybrid mass spectrometer (LTQ-Orbitrap Velos Pro, Thermo Scientific) via a nanoelectrospray ion source (Thermo Scientific). The spray voltage was set to 2.2 kV, and the temperature of the heated capillary was set to 200 °C. Full-scan MS survey spectra (m/z 335–1800) in profile mode were acquired in the Orbitrap with a resolution of 60 000 after accumulation of 1 000 000 ions. A lock mass of 445.120 024 was enabled for survey scans to improve mass accuracy. The 15 most intense peptide ions from the preview scan in the Orbitrap were fragmented by collision-induced dissociation (normalized collision energy, 35%; activation Q, 0.250; and activation time, 10 ms) in the LTQ after the accumulation of 10 000 ions. Dynamic exclusion parameters were set as follows: repeat count, 1; repeat duration, 30 s; exclusion list size, 500; exclusion duration, 45 s; exclusion mass width, plus/minus 10 ppm (relative to reference mass). Maximal filling times were 10 ms for the full scans and 100 ms for the MS/MS scans. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged species were rejected. Data
were acquired using Xcalibur software.

The following analyses described were performed by Laura Graham at the Roslin Institute. Raw proteomic data were imported into Progenesis for characterization and analysis of relative ion abundance. 2D representations of MS/MS output were created for each sample and these were aligned to determine similar features (average alignment score >80%). Following alignment, data was filtered by retention time with features detected below 12.16 minutes and above 133.78 minutes discarded to correct for elution variability. The runs were grouped according to age and mitochondrial subcellular localization and Statistical P values were automatically generated in Progenesis software through a 1-way ANOVA on the ArcSinh transform of the normalized data.

Peptides were filtered by the following criteria: power <0.8, fold change >1.2, p>0.05 and the remaining data were exported from Progenesis for identification of individual peptide sequences using the IPI-<i>mus musculus</i> database via Mascot Search Engine (V2.3.2). Enzyme specificity was set to that of trypsin, allowing for cleavage N-terminal to proline residues and between aspartic acid and proline residues. Other parameters used were as follows. (i) Variable modifications: methionine oxidation, methionine dioxidation, protein N-acetylation, gln → pyro-glu. (ii) Fixed modifications: cysteine carbamidomethylation. (iii) MS/MS tolerance: FTMS- 10 ppm, ITMS- 0.6 Da. (iv) Minimum peptide length: 6. (v) Maximum missed cleavages: 2. (vi) False discovery rate: 1%. A cutoff score of >34 was used based on Mascot probability threshold of 0.05 that the observed hit is a random event. As an indication of identification certainty, the false discovery rate for peptide matches above identity threshold was set at 1%.

Identified proteins were re-imported into Progenesis for further processing. Proteins were subject to stringent filtering parameters to eliminate those which had <2 unique peptides and p>0.05 to obtain the proteins which demonstrated statistically significant alterations in mitochondrial protein expression over the ageing time course.
4.2.5 Biolayout Express\textsuperscript{3D}

Proteomic data was dissected using the complex pattern recognition software, Biolayout Express\textsuperscript{3D} (159). The software allows visualization of molecular networks by applying Markov clustering algorithms to raw proteomic data (MCL 2.2). All graphs were clustered using Pearson correlation $r=0.96$. Clusters of interest indicating age-dependent alterations included those that demonstrated a steady up- or down-regulation or a late stage up- or late stage down-regulation during the timecourse of ageing. Proteins from clusters with analogous expression profiles underwent a subtractive process – candidates appearing in both the synaptic and non-synaptic clusters, altered in the same manner, were unlikely to regulate synaptic stability during ageing and were eliminated from further analyses.

4.2.6 Drosophila Stocks

See methods section 2.11 for a comprehensive overview. All stocks lines used for experiments were obtained from the BDSC: 9837, 26650, 42580 (see Table 3). Flies were raised on standard cornmeal food at room temperature. Homology of mouse gene of interest and Drosophila ortholog was determined by input into DIOPT (DRSC Integrative Ortholog Prediction Tool (162): see Table 3). The elav-Gal4 driver strain was used for all experiments (see Table 4). Immunohistochemistry methods are described in chapter 2, section 2.12.
4.3 Results

4.3.1 Spatiotemporal characterisation of discrete mitochondrial proteomes.

To determine age-dependent molecular alterations occurring in discrete neuronal compartments of the rodent brain, we purified and characterised isolated mitochondria from cortical synaptic and non-synaptic compartments at 3 time points (young adult, mid-age, old). Quantitative label-free proteomic analyses identified >1800 common proteins common to each subpopulation across the time-course (Fig. 20A&B), revealing dynamic variations in synaptic protein expression. Markedly, over 1000 proteins were altered by greater than 20% in each discrete mitochondrial population, demonstrating significant age-dependent modifications at the proteome level. To assess the purity of mitochondrial preparations, PANTHER Gene Ontology bioinformatics software was employed. Input of the 1857 identified proteins from the proteomic data indicated enrichment of the ATP synthase complex and the mitochondrial inner membrane suggesting relative purity of the preparations.

<table>
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<tr>
<th>PANTHER GO-Slim Cellular Component</th>
<th>Mus musculus gene number</th>
<th>Fold Enrichment</th>
<th>P-value</th>
</tr>
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<td>1.26E-03</td>
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<td>Mitochondrial inner membrane</td>
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<td>5.33</td>
<td>1.99E-07</td>
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<td>SNARE complex</td>
<td>39</td>
<td>4.81</td>
<td>2.73E-05</td>
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<td>Vesicle coat</td>
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<td>2.42E-04</td>
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<tr>
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</tr>
<tr>
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<td>3.67E-02</td>
</tr>
<tr>
<td>Actin cytoskeleton</td>
<td>234</td>
<td>2.76</td>
<td>3.52E-03</td>
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</table>
Figure 20: Spatiotemporal characterisation of the mitochondrial proteome. A & B. Venn diagrams demonstrating identification of synaptic and non-synaptic mitochondrial proteins. Proteins were identified and filtered in Progenesis software using the criteria \( p > 0.05 \) and 1 unique peptide. 1857 common proteins were identified in both the synaptic and non-synaptic mitochondrial datasets and these were utilized for all further analyses. C. PANTHER GO-Slim cellular component enrichment analysis of the synaptic and non-synaptic mitochondrial raw data. Table indicates enrichment of the mitochondrial ATP synthase complex and mitochondrial inner membrane suggesting relative purity of preparations. Fold-enrichment values greater than 1 denote overrepresentation of category in dataset.

4.3.2 Mitochondrial ageing demonstrates subcellular heterogeneity.

To address whether dynamic variations in the mitochondrial proteome may be contributing to age-dependent synaptic vulnerability, we sought to determine if organelles from discrete subcellular compartments aged in a similar manner. Using the objective network visualisation software Biolayout Express\textsuperscript{3D} ((159) www.biolayout.org), we generated synaptic and non-synaptic mitochondria principal component analysis (PCA) correlation graphs demonstrating relative age-dependent similarities (Fig. 21A&B). Examination of these networks confirmed distinct compartment-dependent clustering profiles. Non-synaptic mitochondria display a single network suggesting a degree of homogeneity between young, mid-age and old samples (Fig. 21B). Though the old and young mitochondria appear to demonstrate reduced equivalence – represented by the distance between population nodes – the data suggest that there are fewer significant temporal alterations occurring in the non-synaptic versus the synaptic organelles. Conversely, the synaptic mitochondria demonstrate increased heterogeneity between sample populations. Fragmentation of the graph indicates that the aged synaptic mitochondrial population possesses discrete protein expression profiles (Fig. 21A), which may contribute to the enhanced vulnerability of cortical synapses at this particular age. Thus, there are indications that isolated mitochondrial populations derived from discrete subcellular compartments age heterogeneously and the unique alterations occurring in synaptic mitochondria may dictate the potential vulnerability of synapses.
Figure 21: Mitochondrial ageing demonstrates subcellular heterogeneity. Unbiased sample-sample correlation analysis generated from BioLayout Express3D software. Nodes signify individual samples and edges reflect the strength of correlation of expression. YS – young synaptic mitochondria; MS – mid-age synaptic mitochondria; OS – old synaptic mitochondria; YNS – young non-synaptic mitochondria; MNS – mid-age non-synaptic mitochondria; ONS – old non-synaptic mitochondria. All graphs clustered using Pearson r=0.96.

4.3.3 Temporal protein profiling of discrete mitochondrial populations reveals protein expression trends correlating with synaptic vulnerability.

In conjunction with the principal component analyses, to examine compartment-specific temporal mitochondrial alterations further, we generated network graphs of the synaptic and non-synaptic mitochondrial timecourse proteomic data, again utilising Biolayout Express3D (159). The software applies unbiased Markov clustering algorithms to the input data and groups proteins displaying similar expression trends, allowing visualisation of spatiotemporal profiles and identification of discrete biochemical cascades altered within the dataset.
Synaptic and non-synaptic mitochondrial graphs were constructed utilizing the 1857 commonly identified proteins through the timecourse of ageing (see Fig. 20A&B), providing 35-45 clusters per subpopulation. Interestingly, in contrast to the principal component analyses, the synaptic mitochondrial timecourse exhibited one large interconnected network whereas the non-synaptic displayed fragmentation into a multitude of smaller arrangements (Fig. 22A&B). Although the fragmentation of the non-synaptic mitochondrial protein timecourse may be postulated to be due to heterogeneity between the young, mid-age and old sample populations, this is incongruous with the PCA. Instead, the fragmentation is likely occurring due to the variability in individual protein expression. Numerous individual proteins harbor unique expression profiles throughout the timecourse, which prevents assignment to a cluster and reduces the connectivity between nodes (Fig. 22B). Conversely, the synaptic mitochondria timecourse displays a multitude of proteins that exhibit similar expression profiles, despite the heterogeneity between age groups, thus enhancing the number of candidates assigned to clusters and connectivity between nodes (Fig. 22A).

Figure 22: Ageing mitochondrial subpopulations exhibit distinct protein clustering patterns. Protein-protein correlation networks displaying protein expression through the
timecourse of ageing in mitochondrial subpopulations. Nodes signify individual proteins and edges reflect the strength of correlation of expression. Colours represent clusters of proteins that are grouped together based on their expression profiles. All graphs clustered using Pearson r=0.96.

By utilising two mitochondrial subpopulations demonstrating disparate profiles of ageing, we aimed to identify potential mitochondrial regulators of synaptic vulnerability with comparison of analogous protein expression profiles. Clusters displaying particular expression trends of interest were selected from both the synaptic and non-synaptic mitochondrial timecourse network graphs generated in Biolayout Express3D (Fig. 22A&B). Those clusters exhibiting steady up- or down-regulation protein expression profiles during the timecourse were considered as potential biomarkers of normal healthy ageing due to the predictable age-dependent tractability of those candidates (Fig. 23A). Conversely, proteins displaying late-stage increases or decreases in expression were regarded as potential biomarkers of synaptic and/or organelle vulnerability as the abrupt expression changes observed in the old synaptic and non-synaptic populations likely reflected acute alterations disrupting mitochondrial homeostasis (Fig. 23B). In order to identify mitochondrial candidates that may be regulating cortical synaptic vulnerability during ageing, proteins from synaptic and non-synaptic mitochondrial clusters with analogous expression profiles were subject to a subtractive process. Proteins demonstrating equivalent spatiotemporal profiles in both synaptic and non-synaptic mitochondrial populations were not considered to be regulators of cortical synaptic vulnerability and filtered from the data prior to further analysis (Fig. 23C&D). This provided 451 differentially expressed mitochondrial proteins that may be associated with age-dependent synaptic vulnerability (Fig. 23A&B).
A. Biomarkers of mitochondrial aging

B. Biomarkers of mitochondrial vulnerability

C. Synaptic Non-synaptic

D. Synaptic Non-synaptic

E. Mitochondria Non-synaptic

F. Mitochondria Synaptic

G. Protein expression

H. Late-stage decrease

I. Late-stage increase

J. Steady decrease

K. Steady increase
Figure 23: Temporal profiling of distinct mitochondrial subpopulations reveals biomarkers of organelle ageing and vulnerability. A. Biomarkers of ageing: example temporal expression profiles of proteins demonstrating a steady up- or down-regulation during ageing. B. Biomarkers of vulnerability: example clusters displaying late stage increases or decreases in temporal protein expression. All graphs were generated in BioLayout Express$^{3D}$ ($r=0.96$) and display the mean protein expression across the timecourse in mitochondrial samples. C&D. Venn diagrams indicating subtraction of candidates. Proteins demonstrating equivalent spatiotemporal profiles in both synaptic and non-synaptic mitochondria (shown at intersection) were not considered to be regulators of synaptic vulnerability and were subtracted from further analysis.

4.3.4 Temporal regulation of the synaptic mitochondrial proteome modulates synaptic morphology

Despite characterizing the global spatiotemporal changes occurring in discrete mitochondrial subpopulations, it remained unclear whether divergence in expression of individual mitochondrial candidate proteins may be capable of actively regulating synaptic vulnerability. To elucidate mitochondrial regulators of synaptic stability, we initially mapped the individual temporal expression profiles of the 451 candidates identified by the Biolayout Express$^{3D}$ analyses using Python Jupyter Notebook (see Appendix 2). Upon examination of the protein expression trends, we hypothesized that candidates likely capable of modulating synaptic stability would exhibit unequivocal temporal profiles across mitochondrial subpopulations, with considerable divergence at old age. Thus, proteins with corresponding expression at the young and mid-age time-points in both synaptic and non-synaptic mitochondria, followed by a significant demarcation in expression at old age were selected as potential regulators of synaptic vulnerability. These particular expression profiles correlate with previous reports of significant age-dependent alterations in mitochondrial morphology (206), bioenergetics (187) and calcium buffering capacities (207), which have the propensity to perturb synaptic structure and function (208).
Following the identification of 243 proteins demonstrating the archetypal spatiotemporal expression profile (Fig. 24B), we further refined the candidate compendium by selecting proteins that exhibited a >2 fold-change in expression between synaptic and non-synaptic mitochondria at the old-age time point (Fig. 24B). The remaining 96 candidates represented potential mitochondrial regulators of age-dependent synaptic vulnerability. Interestingly, DAVID enrichment analyses of these mitochondrial candidates indicated that alterations in DNA methylation cascades may be contributing to synaptic vulnerability during advancing age. To examine whether alterations in candidate protein expression in aged synaptic mitochondria was contributing to the vulnerability status of synaptic compartments \textit{in vivo}, we utilized a molecular genetic approach at the \textit{Drosophila} larval NMJ to assess the regulatory role of the individual candidates Rab31, RhoG and Mcu at the synapse.
Figure 24: Identification of mitochondrial-associated candidates regulating synaptic stability during ageing. A. Heat map displaying average normalized abundance values of 451 differentially expressed mitochondrial candidates associated with alterations in synaptic stability during ageing. Note the divergence in protein expression in the old synaptic mitochondria. Blue indicates low expression and red high expression. YS - young synaptic; MS – mid-age synaptic; OS – old synaptic; YNS – young non-synaptic; MNS – mid-age non-synaptic; ONS – old non-synaptic. B. Scatterplot indicating the hierarchical filtering of
proteins by stringent parameters for identification of mitochondrial candidates regulating synaptic stability. Left panel displays 451 tractable proteins identified from Biolayout Express\textsuperscript{3D} analyses. Middle panel represents filtering of 451 candidates by archetypical protein expression profile (as illustrated in Appendix 2). Right panel exhibits the 96 mitochondrial-associated candidates displaying archetypical protein profiles and a 2-fold change between mitochondrial subpopulations at the old age time point. Red lines indicate 2-fold change. C. DAVID enrichment analysis of 96 identified candidates that may have the propensity to modulate synaptic stability during ageing. D. Candidate protein expression profiles. Graphs represent temporal expression profiles of identified candidates in both synaptic and non-synaptic mitochondria. Note the similar expression profiles through young and mid-age in both synaptic and non-synaptic mitochondria followed by significant divergence in expression at old age. All graphs display the ratio of candidate protein expression against the young age. Synaptic mitochondrial temporal protein expression - grey; non-synaptic temporal protein expression - black.

Recapitulation of candidate protein expression at the larval NMJ was achieved using the UAS/Gal4 system, promoting pan-neuronal expression of the selected transgenes under control of the elav-Gal4 driver (see Table 3 for Drosophila orthologs). Regulated expression of the constructs (9837, 26650, 42580) resulted in viable offspring from all crosses, allowing examination of individual candidate modulatory effects on the synaptic architecture. Manipulation of Rab31 (9837), RhoG (26650) and Mcu (42580) expression revealed striking synaptic phenotypes at the third instar larva muscle 12/13 NMJ in multiple morphological parameters associated with the structural and functional stability of the synapse (Fig. 25). Indeed, enhanced constitutive expression of the mitochondrial associated vesicular trafficking protein Rab31 promoted modest reductions in the total bouton area (p<0.05) in addition to concomitant decreases in the active zone punctate size (p<0.0001) versus the control line (Fig. 25A-C). Although Rab31 NMJ arborisation appears to demonstrate considerable similarity to that of the control, minor reductions in the branching of distal processes may be contributing to the observed reductions in total bouton area. Conversely, RhoG overexpression promotes marked perturbations in arborisation, with NMJs displaying truncated branches and aberrant compacted morphologies (Fig. 25A). Correspondingly, significant reductions in bouton volume (p<0.0001)
and active zone punctate size (p<0.0001) are observed (Fig. 25A-C), which are likely mediated by the role of RhoG in polymerization of the actin cytoskeleton and regulation of dendritic differentiation and stabilization during development (209). RNAi-mediated knock-down of Mcu expression produced a morphologically distinct but quantitatively similar phenotype to that of RhoG manipulation (Fig. 25A). Reductions in Mcu activation also appear to attenuate the stereotypic arborisation patterns of the NMJ, in addition to facilitating decreases in the total bouton area (p<0.0001) and active zone punctate size ((p<0.0001) Fig. 25A-C). Despite all candidate lines (Rab31, RhoG and Mcu) demonstrating a significant reduction in active zone punctate size, with quantification of total active zone staining per NMJ, we report no significant difference versus control lines. Although vesicular dynamics may be regulated via biochemical cascades independent of those manipulated here, the significant reduction in total bouton area in multiple candidate lines suggests that coordinated expression of these particular mitochondrial proteins may be essential for the continued maintenance of synaptic-specific structures during advancing age.
Figure 25: Recapitulation of candidate protein expression promotes aberrant synaptic phenotypes at the *Drosophila* NMJ. A. Representative confocal images of muscle 12 NMJs, labelled with anti-HRP and anti-BRP. Overexpression of Rab31 and RhoG and RNAi-mediated knock-down of Mcu promote aberrant synaptic phenotypes at the NMJ. Lower panel displays masks utilized for quantification of morphological parameters. B. Graphs represent quantification of control and candidate line total bouton area (µm). Rab31 (* p = 0.0376), RhoG (**** p<0.0001) and Mcu (**** p<0.0001) demonstrate significant reductions in total bouton area. C. Graphs indicate the average size of active zone punctate (µm) in control and candidate lines. Rab31, RhoG and Mcu all display a significant decrease in punctate size versus control (**** p<0.0001). D. Graphs display the total area of active zone staining in control and candidate lines. Rab31 (p = 0.926), RhoG (p = 0.3574) and Mcu
(p = 0.5397) demonstrate no significant difference in active zone area per NMJ. All lines used the elav-Gal4 driver system. NMJs imaged at 63x. Scale bar = 10µm, n=5. Statistical analyses utilized unpaired two-tailed Student’s t-test (* p = 0.05; ** p = <0.01; *** p = <0.001; **** p = <0.0001).
4.4 Discussion

How the spatiotemporal regulation of the mitochondrial proteome and synaptic function intersect during advancing age is poorly understood. Here, we indicate that selective biochemical alterations in the synaptic mitochondrial proteome may promote age-related perturbations in synapse structure and function in vivo. Temporal proteomic profiling of distinct subcellular mitochondrial populations from the rodent cortex revealed discrete and dynamic alterations in both the synaptic and non-synaptic mitochondrial proteomes during normal healthy ageing. Intriguingly, aged synaptic mitochondria appeared to harbour a unique proteome, exhibiting significant divergence in protein expression. Recapitulation of aged synaptic mitochondrial protein expression using molecular genetic tools in vivo revealed several novel functional mediators that have the propensity to significantly modulate multiple morphological parameters at the synapse, suggesting that the mitochondrial proteome and synaptic morphometry are intimately intertwined. The data indicate that selective alterations in synaptic mitochondrial protein expression may, in part, mediate enhanced vulnerability of the cortical synapse during advancing age.

4.4.1 Mitochondrial candidates modulating synaptic morphology

The identification of 96 candidates demonstrating a >2 fold change between synaptic and non-synaptic mitochondria in a temporal profile associated with increased vulnerability of the synapse is highly indicative of the regulatory role the organelles may play in modulating synaptic structure and function during ageing. Interestingly, the 96 differentially expressed candidates appeared to demonstrate enrichment in DNA methylation cascades. Recent evidence has suggested that the methylation of mtDNA is age- and brain region dependent (210, 211) and reductions in mtDNA methylation promote transcriptional changes associated with senescent phenotypes in vitro (212). Furthermore, decreases in mtDNA methylation have also been associated with genomic instability, promoting mutagenesis and dysregulation of the respiratory complex genes encoded by the mitochondrion (210). Strikingly, the data obtained in this study indicate that mtDNA methylation may also demonstrate discrete subcellular patterns, which suggests differential regulation of mtDNA integrity in
distinct mitochondrial subpopulations. Although global perturbations in mtDNA stability have been widely associated with advancing age, few studies have examined whether mtDNA integrity differs in ageing synaptic and non-synaptic mitochondria. Our results suggest that this may warrant exploration to determine if synaptic mitochondria exhibit increased susceptibility to mtDNA mutagenesis, organelle dysfunction and concomitant compartmental instability during ageing.

Though we identified 96 candidates that represented potential mitochondrial regulators of age-dependent synaptic vulnerability, due to time constraints, we were only able to phenotypically assess 3 candidates in vivo. Despite this, we demonstrate that recapitulation of Rab31, RhoG and Mcu protein expression at the Drosophila larval NMJ promotes aberrant synaptic phenotypes, including reductions in total bouton area and decreases in active zone puncta size. Of particular interest is the decrease in bouton active zone size in all candidate lines. Indeed, recent evidence suggests that the degree of BRP staining present at individual active zone sites correlates with the quantity of readily releasable vesicles and thus the probability of synaptic vesicle release (213). Accordingly, the results obtained from this study indicate that misexpression of selected mitochondrial candidates may promote functional alterations in synaptic vesicle recycling and neurotransmission dynamics – processes that have been widely associated with age-dependent alterations in synaptic stability. Interestingly, these data are in agreement with the Hara et al study (refer to section 4.1), which describes age- and mitochondrial-morphology dependent decreases in the presynaptic active zone size and the number of synaptic vesicles in NHP cortical synapses (190). Though we report significant reductions in the size of bouton active zone puncta, quantification of the global active zone area per NMJ suggested no differences between control and candidate lines. Although speculative, this may represent a compensatory mechanism adopted by the cell to enable the maintenance of transmission and plastic properties at the synapse.

The localisation and functional specialisation of the identified candidates is wide-ranging, likely reflecting the convergence of numerous mitochondrial mechanistic pathways required for homeostatic regulation of the synaptic compartment. Rab31 is
a small GTP-binding molecule localized to the mitochondrion, golgi and plasma membrane and appears to regulate vesicular targeting, mobilization and docking (214-216). Though there are relatively few studies documenting the constitutive role of the protein and how misexpression may modulate cellular structural and functional properties, evidence indicates that Rab31 is a member of the Rab5 superfamily of Rab GTPases (216). Overexpression of the Rab5 family has been directly associated with impairments in synaptic vesicle recycling and neurotransmission at the *Drosophila* NMJ (217, 218), although the mechanism governing this impairment remains elusive. Thus, it is probable that the age-dependent overexpression of Rab31 in synaptic mitochondria may modulate the size of the bouton active zone through a corresponding cascade, promoting concomitant synaptic dysfunction via alterations in transmission, plasticity and vesicular dynamics. Similarly, overexpression of the mitochondrial-associated protein RhoG (219) also demonstrated a reduction in bouton active zone size in addition to a highly significant reduction in total bouton area. RhoG exhibits involvement in the polymerization of the actin cytoskeleton and regulation of dendritic and axonal branching and stabilization, particularly during development (210). Previous studies examining overexpression of RhoG report reduced axonal and dendritic complexity *in vivo* (220), with significant alterations in arborisation, which appear to correlate with the compacted phenotype we present here. To our knowledge, there are currently no studies describing the role of RhoG at the synaptic terminal during ageing, thus we present novel data demonstrating that temporal increases in synaptic mitochondrial RhoG expression, alter multiple morphological parameters associated with compartmental function and stability. Though the RhoG and Mcu lines exhibited quantitatively and morphologically similar phenotypes at the NMJ, divergent mechanisms likely mediate the reported synaptic alterations. Mcu is a mitochondrial calcium uniporter that regulates intracellular Ca$^{2+}$ concentrations via uptake of ions into the mitochondrial matrix. Due to the significant fluctuations in presynaptic Ca$^{2+}$ concentrations during neurotransmission events, the mitochondrial Mcu has previously been associated with structural and functional perturbations in the synaptic milieu during ageing and pathogenesis (221-223); however, investigations examining the effects of Mcu knock-down *in vitro* report conflicting
results. There are reports that reductions in Mcu expression diminishes vesicular mobility and release, promoting concomitant alterations in short-term synaptic plasticity (221), which suggests that regulated Mcu expression facilitates neurotransmission properties via presynaptic Ca\textsuperscript{2+} clearance cascades. However, studies examining synaptic vesicle recycling kinetics document no effect of Mcu knock-down on presynaptic vesicular exocytosis, total active zone area or intracellular Ca\textsuperscript{2+} levels (222). Though we have not investigated the functional properties of the Drosophila NMJs harbouring reductions in Mcu expression, our data indicate that Mcu may modulate the size of the bouton active zone, which may affect the probability of vesicular release due to a smaller pool of readily releasable vesicles. However, whether this is due to elevations in presynaptic intracellular Ca\textsuperscript{2+}, reductions in Ca\textsuperscript{2+}-dependent ATP production or alterations in mitochondrial dynamics remains undetermined.

4.4.2 Troubleshooting strategies
Although we have provided a thorough spatiotemporal characterisation of the synaptic and non-synaptic mitochondrial proteomes, identifying several biochemical substrates that may functionally regulate the stability of the mitochondria and the synaptic milieu during advancing age, the investigation initially proved highly problematic. We were unable to biochemically validate the purification of the mitochondrial preparations or the accuracy of the proteomic data using traditional techniques due to the addition of interfering reagents into the label-free sample buffer. The presence of 0.1 M d/l-dithiothreitol (DTT) in all samples prevented quantitation of total protein concentration despite the use of a number of methods, including micro-BCA and Bradford assays. Attempts to precipitate the DTT out of the samples were also unsuccessful. Furthermore, we encountered a number of mass spectrometry issues, preventing the reliable identification of proteins in the young and mid-age synaptic mitochondrial samples (Appendix 3). We suggest that this was due to the presence of polymers within the samples, likely caused by transference from the tube. Fortunately, reprocessing of the samples and the addition of new synaptic and non-synaptic mitochondrial tissues from animals of equivalent ages ameliorated the reported mass spectrometry issues and enabled identification and
analysis of the proteins from these samples (data shown throughout this chapter). Despite this, the duration of time spent attempting to decipher the numerous issues highlighted was significant, detracting from the potential collection of additional in vivo data, including characterizing the morphologies of synaptic and non-synaptic mitochondria with MitoTracker in the Drosophila lines harbouring misexpression constructs.

4.4.3 Conclusions
To our knowledge, this is the first study documenting the spatiotemporal alterations occurring in both synaptic and non-synaptic mitochondrial populations during advancing age. Cumulatively, our data describe that selective alterations in the aged synaptic mitochondrial proteome modulate multiple morphological parameters associated with synaptic dysfunction in vivo. Further investigations exploring how dynamic alterations in the synaptic mitochondrial proteome may attenuate neurotransmission, plasticity and vesicular mobility are required to enable elucidation of the mechanistic pathways orchestrating age-dependent impairments in synaptic function.
Chapter 5. TGFβ1 regulates regional synaptic vulnerability during normal healthy ageing

I confirm that I have authored the following text and that the work contained within this chapter is currently under consideration at Nature Neuroscience (August 2017)

5.1 Introduction

The loss and dysfunction of selected populations of synapses is characteristic of mammalian brain ageing and alterations in these receptive compartments are considered to underpin age-related cognitive decline (1-6). It has been well established that the hippocampal synaptic architecture displays particular vulnerability to a wide range of stimuli during advancing age (1-3), whereas populations of synapses resident to the occipital cortex exhibit resistance to age-related alterations (4-13). Although the synaptic alterations that underlie age-related cognitive decline differ from the extensive neuronal loss that leads to dementia and Alzheimer’s disease (AD), these alterations may render neurons more vulnerable to degeneration during the ageing process (1, 14). An essential area for investigation is to determine how synaptic alterations may leave a neuron vulnerable to neurodegeneration and the pathological substrates promoting such vulnerability. A great deal of our current knowledge concerning the mechanisms of cognitive and brain ageing has been provided by rodent studies. Several investigations have reported heterogeneous expression of the hippocampal synaptic proteome in ageing rodents (15-17), however it has recently been established that relatively few age-related gene and protein expression alterations demonstrate conservation from mouse to man (18). Evolutionary divergence of the primate cortex has promoted functional neuronal alterations that are uniquely primate (1) and studies documenting rodent biological brain ageing are not necessarily representative of the complex processes occurring in the human patient, particularly with regards to selective synaptic vulnerability (18). Thus, in order to tease apart human age-related cognitive decline,
non-human primates provide some advantages. Non-human primates (NHP) are phylogenetically closer to humans and possess distinctly primate morphological, endocrine, behavioural, and cognitive traits (1, 6), in addition to an increased lifespan, which may provide data uniquely relevant to human ageing.

In what appears to be the first investigation into the regional diversity of the primate synaptic proteome during the adult lifespan, we present a comprehensive ‘synaptic atlas’ supporting the notion that local biochemical alterations dictate selective synaptic vulnerability. Here, we employ an unbiased combinatorial approach, including quantitative proteomic analyses coupled with in vivo candidate assessments in lower order animals (Drosophila), to temporally profile primate brain regional synapse biochemistry during normal healthy ageing. We demonstrate that synaptic ageing is brain-region dependent and discrete populations of synapses significantly differ at a biochemical level in the healthy human and non-human primate brain. Recapitulation of aged hippocampal protein expression with genetic manipulation in vivo revealed several novel candidates that have the propensity to significantly modulate multiple morphological parameters at the synapse. Furthermore, we demonstrate that several of these candidates sit downstream of TGFβ1 and activation of the TGFβ1 signalling cascade in hippocampal synaptic populations drives the aberrant expression of selected candidates during ageing. Finally, we show that selective pharmacological inhibition of this pathway rescues synaptic phenotypes in multiple candidate lines. The current investigation has affirmed that activation of the TGFβ1 transduction pathway modulates synaptic stability and thus may contribute to the selective vulnerability of hippocampal synapses during ageing. Additionally, the thorough ‘molecular mapping’ of healthy primate brain ageing has provided a dynamic temporal synaptic atlas that we may compare disease processes to, allowing elucidation of biochemical alterations that may be regarded as pathogenic.
5.2 Methods

5.2.1 Ethics

In compliance with the 3Rs, no animals were bred specifically for this project. All tissue samples used in the current study were derived from existing archived brains or harvested alongside other ongoing experiments. The Oregon Health & Sciences University Institutional Animal Care and Use Committee at the Oregon National Primate Research Center (ONPRC) and the University of Edinburgh internal ethics committee approved all animal experiments.

5.2.2 Animals

12 rhesus macaques (*Macaca mulatta*) of differing sexes and ages were utilized for the studies described. Animals were assigned to the young adult (mean age = 9.5 years), mid-age (mean age = 15.6 years) or old age (mean age = 23 years) group, with 4 animals per time point. All animals were euthanized according to procedures recommended by the 2013 Edition of the American Veterinary Medical Association *Guidelines for the Euthanasia of Animals*. Each animal was sedated with ketamine, administered pentobarbital (30 mg/kg, i.v.), and exsanguinated by severance of the descending aorta. Brains were removed and appropriate regional dissections performed before freezing the samples in liquid nitrogen.

5.2.3 Human patient samples

Human patient samples were obtained from the Edinburgh Brain Bank. All tissues were classified as controls due to the absence of gross pathological hallmarks and neurological disease. Human tissues were assigned to equivalent age groups: young (18-25 years), mid-age (40-50 years) or old age (70+ years), with 4 samples per time point. Use of human tissue for post-mortem studies was reviewed and approved by the Edinburgh Brain Bank ethics committee. The Edinburgh Brain Bank is a Medical Research Council funded facility with research ethics committee approval (11/ES/0022).
5.2.4 Synaptosomal preparations
All NHP tissue preparations were performed by Laura Graham at Oregon National Primate Research Center, Oregon Health and Sciences University, OR, USA (September 2014). Regional brain tissue samples were homogenised in an ice-cold isotonic sucrose solution (0.32 M sucrose, 1 mM EDTA, 5 mM Tris-HCl, pH 7.4). Homogenates were centrifuged in a fixed-angle rotor at 900 g for 10 min and the supernatant (S1) was collected. The pellet (P1) was resuspended in sucrose solution and centrifuged again at 900 g for 10 min. Supernatants were combined and centrifuged in a fixed angle rotor at 20,000 g for 15 min. The remaining pellets (P2) contained the synaptosomes.

5.2.5 Protein concentration assay
Samples were homogenized in SDT lysis buffer + 1% protease cocktail inhibitor (Thermo Scientific). After homogenisation, samples were centrifuged at 20,000g for 20 minutes at 4°C. The supernatant containing the solubilised protein was removed and pellets discarded. Protein concentration of samples was determined using a Pierce Micro BCA assay kit according to the manufacturers instructions.

5.2.6 Label-free proteomics
Mass spectrometry was performed by Dr. Michael Naldrett at the Donald Danforth Plant Science Center, St. Louis, Missouri, USA. Regional synaptosomal preparations were extracted in SDT lysis buffer containing 100 mM Tris-HCl (pH 7.6) and 4% (W/V) Sodium dodecyl sulfate (VWR). For efficient protein extraction, lysates were freeze–thawed and homogenized in SDT buffer several times. Protein concentration was then determined using BCA assay. Aliquots (200 µg) of each synaptosomal preparation were processed through FASP (filter-aided sample preparation) involving buffer exchange to 8 M urea and alkylation with 50 mM iodoacetamide prior to a double digestion with trypsin (Roche, sequencing grade), initially for 4 h, then overnight at 30 °C. Trypsin-digested peptides were separated using an Ultimate 3000 RSLC (Thermo Scientific) nanoflow LC system with the column oven set to 35
°C. Technical replicates (3 × ~1 µg) of each sample were loaded at a constant flow of 5 µL/min onto a trapping cartridge (PepMap100, C18, 5µm, 100Å 0.3 x 5 mm; Thermo Scientific, San Jose, CA) using 2% Acetonitrile, 0.1 % formic acid. After trap enrichment, peptides were separated on a peptide CSH, 1.7µm, 130Å, 75µm x 250mm C18 column (Waters Corp, Milford, MA) with the following gradient: t=0 min, 2 % B; t=6, 2 % B; t=20, 8 % B; t=110, 24 % B; t=135, 37 % B where solvent A is water with 0.1 % formic acid and solvent B is 80% acetonitrile with 0.1% formic acid, with a constant flow of 260 nL/min. The HPLC system was coupled to a linear ion trap Orbitrap hybrid mass spectrometer (LTQ-Orbitrap Velos Pro, Thermo Scientific) via a nanoelectrospray ion source (Thermo Scientific). The spray voltage was set to 2.2 kV, and the temperature of the heated capillary was set to 200 °C. Full-scan MS survey spectra (m/z 335–1800) in profile mode were acquired in the Orbitrap with a resolution of 60 000 after accumulation of 1 000 000 ions. A lock mass of 445.120 024 was enabled for survey scans to improve mass accuracy. The 15 most intense peptide ions from the preview scan in the Orbitrap were fragmented by collision-induced dissociation (normalized collision energy, 35%; activation Q, 0.250; and activation time, 10 ms) in the LTQ after the accumulation of 10 000 ions. Dynamic exclusion parameters were set as follows: repeat count, 1; repeat duration, 30 s; exclusion list size, 500; exclusion duration, 45 s; exclusion mass width, plus/minus 10 ppm (relative to reference mass). Maximal filling times were 10 ms for the full scans and 100 ms for the MS/MS scans. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged species were rejected. Data were acquired using Xcalibur software.

The following analyses described were performed by Laura Graham at the Roslin Institute. Raw proteomic data were imported into Progenesis for characterization and analysis of relative ion abundance. 2D representations of MS/MS output were created for each sample and these were aligned to determine similar features (average alignment score >80%). Following alignment, data was filtered by retention time with features detected below 17 minutes and above 140 minutes discarded to correct for elution variability. The runs were grouped according to age and brain region and Statistical P values were automatically generated in Progenesis software.
through a 1 way ANOVA on the ArcSinh transform of the normalized data.

Peptides were filtered by the following criteria: power <0.8, fold change >1.2, p>0.05 and the remaining data were exported from Progenesis for identification of individual peptide sequences using the IPI-\textit{macaca mullata} database via Mascot Search Engine (V2.3.2). Enzyme specificity was set to that of trypsin, allowing for cleavage N-terminal to proline residues and between aspartic acid and proline residues. Other parameters used were as follows. (i) Variable modifications: methionine oxidation, methionine dioxidation, protein N-acetylation, gln $\rightarrow$ pyro-glu. (ii) Fixed modifications: cysteine carbamidomethylation. (iii) MS/MS tolerance: FTMS- 10 ppm, ITMS- 0.6 Da. (iv) Minimum peptide length: 6. (v) Maximum missed cleavages: 2. (vi) False discovery rate: 1%. A cutoff score of >34 was used based on Mascot probability threshold of 0.05 that the observed hit is a random event. As an indication of identification certainty, the false discovery rate for peptide matches above identity threshold was set at 1%.

Identified proteins were re-imported into Progenesis for further processing. Proteins were subject to stringent filtering parameters to eliminate those which had <2 unique peptides, <1.2 fold change between age groups and p>0.05 to obtain the proteins which demonstrated the largest significant variation in expression over the ageing time course in each brain region.

\textbf{5.2.7 Biolayout Express$^\text{3D}$}

Proteomic data was dissected using the complex pattern recognition software, Biolayout Express$^\text{3D}$ (19). The software allows visualization of molecular networks by applying Markov clustering algorithms to raw proteomic data (MCL 2.2). All graphs were clustered using Pearson correlation r=0.95. Clusters of interest indicating age-dependent alterations included those that demonstrated a steady up- or down-regulation or a late stage up- or late stage down-regulation during the timecourse of ageing. Proteins from clusters with analogous expression profiles underwent a subtractive process – candidates appearing in both the resistant and vulnerable clusters, altered in the same manner, were unlikely to regulate synaptic
vulnerability during ageing. These candidates were eliminated as modulators of synaptic vulnerability.

5.2.8 Ingenuity Pathway Analysis
IPA analyses were performed as previously described (20). See methods section 2.9 for details on the parameters utilized for experimental analysis.

5.2.9 Quantitative Fluorescent Western Blotting
All NHP western blots were performed by Laura Graham at Oregon National Primate Center (October 2016) as previously described (21). Samples were diluted to provide desired protein concentration. 10µg protein was loaded per well into NuPAGE® Novex® 4-12% Bis Tris mini-gels (Life Technologies) and transferred to PVDF membranes using an iBlot® and Invitrogen gel transfer stacks. Membranes were incubated in primary antibodies at 4°C and secondary antibodies at room temperature (concentrations according to manufacturers instructions) before imaging on Li-COR Odyssey infrared scanner. Protein expression was quantified utilising Odyssey software (Li-COR Biosciences). All antibodies utilised are listed in Tables 1 & 2.

5.2.10 Drosophila Stocks
See methods section 2.11 for a comprehensive overview. Flies were raised on standard cornmeal food at room temperature. Homology of rhesus macaque gene of interest and Drosophila ortholog was determined by input into DIOPT (DRSC Integrative Ortholog Prediction Tool (22): see Table 3). The elav-Gal4 driver strain was used for all experiments. Stocks were obtained from the VDRC (v9180; v40466; v22851; 28797; v100773; 34101; v30690; v36166) and Bloomington Drosophila stock center (Canton-S). For pharmacological experiments, 2mM LY364947 (Tocris, cat: 2718) solubilised in 0.3% DMSO (w/v) was added to cornmeal food.

5.2.11 Immunohistochemistry
Third instar larva were selected and dissected in PBS (n=6). The dissected larval
neuromuscular junctions (NMJs) were fixed in Bouin’s fixative (15:5:1 picric acid, 37% formaldehyde and acetic acid) for 10 minutes and washed thoroughly in PBT (PBS + 0.1% TritonX-100). Preparations were blocked in PBT + 10% normal goat serum for 2 hours then incubated in primary antibody overnight at 4°C. NMJs were again washed extensively in PBT and incubated in secondary antibody at room temperature for 2 hours. Samples were mounted on microscope slides using Vectashield mounting medium (Vector Laboratories) and imaged on a Zeiss confocal microscope. Antibodies used are listed in Table 4 & 5.

5.2.12 Statistical Analysis
Data were collected in Microsoft Excel and statistical tests were performed in GraphPad Prism 6 software. For all analyses p<0.05 was considered significant. Statistical tests used are detailed in the results or figure legends where appropriate.
5.3 Results

5.3.1 Characterisation of non-human primate synaptic isolates from discrete brain regions.

Although it has been well documented that discrete neuronal populations demonstrate enhanced vulnerability to insult and degeneration during ageing, the molecular mechanisms governing such processes remain to be elucidated. To determine age-dependent regional molecular alterations occurring in synaptic compartments, we purified and characterised crude synaptosomes from differentially vulnerable brain regions (cerebellum, temporal cortex, occipital cortex, hippocampus) at 3 time points (young adult, mid-age, old). Quantitative label-free proteomic analyses identified >1700 proteins in each region across the time-course, revealing dynamic variations in synaptic protein expression. Strikingly, over 740 proteins were altered by greater than 20% in each discrete region (Fig. 26B), demonstrating significant age-dependent biochemical adaptations. Purity of regional synaptic preparations was verified with quantitative enrichment analyses utilising the raw proteomic data. The normalised average abundance of the well-established synaptic markers synaptic vesicle glycoprotein 2A (SV2A) and synaptotagmin was calculated for each region at the young time point and compared to isolated cortical mitochondria of the same age. To ensure parity between the synaptic samples and the isolated mitochondria, all preparations were loaded onto the mass spectrometer during the same experiment. SV2A and synaptotagmin indicated significant enrichment in all respective regions versus isolated cortical mitochondria suggesting purification of synaptic compartments (Fig. 26C). In addition to demonstrating purification of synaptic fractions, we employed quantitative fluorescent western blotting (QFWB) to determine the veracity of the proteomic data. We observed corresponding protein expression trends in the tissue preparations as indicated by the proteomics for multiple proteins (Fig. 27). Taken together, these results indicate the relative purity of the synaptic preparations and suggest the proteomic data is representative of the molecular alterations occurring in the tissue during ageing.
Figure 26: Regional characterization of the synaptic proteome. A. Schematic illustrating experimental design for comparison of differentially vulnerable brain regions throughout the ageing timecourse (HC = hippocampus; OCC = occipital cortex; TC = temporal cortex; CB = cerebellum). B. Venn diagrams demonstrating regional characterization of the synaptic proteome. Proteins were identified and filtered in Progenesis using the following criteria: p>0.05, <1.2 fold change across the timecourse and 1 unique peptide to obtain the proteins which demonstrate the largest alterations during ageing. Number of proteins up- or down-regulated by >1.2 fold change during ageing, is indicated at the middle intersection. These filtered proteins were utilized for all analyses. C. Purity of regional synaptic preparations. Purity of regional synaptic isolates was verified with quantitative enrichment analyses utilising the raw regional proteomic data and isolated cortical mitochondria. Comparative expression of the synaptic markers SV2A and synaptotagmin indicate synaptic enrichment of all regional preparations. Statistical analyses utilized unpaired two-tailed Student’s t-test (** p = <0.01; *** p = <0.001; **** p = <0.0001).
Figure 27: Validation of regional temporal proteomic data with quantitative fluorescent western blotting. A. Actin loading control for pooled hippocampal and occipital NHP synaptosomes. Samples were pooled according to age group. Bar charts demonstrate there is no significant difference in total protein between ages or regions. B-D. Left bar chart displays the proteomic average normalised expression values of proteins in regional synapses during ageing. Right bar chart demonstrates sample protein expression quantified by fluorescent western blots. Proteomic and sample expression of all proteins (hippocampal NDUFS5, hippocampal OGDH and occipital cortex OGDH) follow the same trend thereby providing validation of the proteomic data. Statistical analysis was performed using an unpaired two-tailed Student’s t-test (* p = <0.05).

5.3.2 The synaptic proteome demonstrates regional heterogeneity during ageing.

To address the question of regional vulnerability, we initially sought to determine whether synapses from discrete brain regions aged in a similar manner. Using the objective network visualisation software Biolayout Express3D (www.biolayout.org),
we generated brain regional principal component analysis correlation graphs demonstrating relative age-dependent similarities (Fig. 28A-D). Examination of these networks confirmed distinct region-dependent clustering profiles. ‘Resistant’ synaptic populations (cerebellum, occipital cortex) exhibited single networks suggesting congruence between young, mid-age and old samples (Fig. 28A&C). Indeed, the cerebellar synapses display significant homogeneity presenting equidistant edges within and between sample populations on the correlation graph (Fig. 28A). Furthermore, similar trends exist in the occipital cortex, with young and mid-age synaptic populations displaying salient similarities. However, the old synaptic population appear to demonstrate reduced equivalence with the mid-age synapses and enhanced similitude with the young samples (Fig. 28C). Conversely, ‘vulnerable’ synaptic populations (temporal cortex, hippocampus) display fragmentation into 2 smaller networks indicating age-dependent heterogeneity (Fig. 28B&D). The temporal cortex demonstrates synaptic isolates derived from young animals appear inherently different from those of later ages (Fig. 28B). Interestingly, the hippocampal correlation graph reveals quite the contrary. Although there appears to be a small degree of variability within and between the young and mid-age samples, it is evident that the aged synaptic population possesses discrete protein expression profiles (Fig. 28D), which may be relevant to the vulnerability status of hippocampal synapses at this particular age. Thus, there are indications that isolated synaptic populations age in a region-dependent manner and these unique alterations may dictate potential vulnerability of synapses.
Figure 28: Synaptic ageing is regionally heterogeneous. Unbiased sample-sample correlation analysis generated from BioLayout Express3D software. Nodes signify individual samples and edges reflect the strength of correlation of expression. All graphs clustered using Pearson $r=0.98$.

5.3.3 Regional profiling of ageing synapses reveals protein expression trends correlating with synaptic vulnerability.

In conjunction with the principal component analyses, to dissect region-specific age-dependent synaptic alterations further, we generated network graphs of the regional timecourse proteomic data, again utilising Biolayout Express3D (19). The software applies unbiased Markov clustering algorithms to the input data and groups proteins displaying similar expression trends. This allows visualisation of spatiotemporal profiles promoting the identification of physiological cascades altered within the dataset. Graphs were constructed utilizing regional differentially expressed proteins (altered $>20\%$) through the timecourse of ageing (see Fig. 26A) providing 20-30 clusters per region. In agreement with the principal component analysis correlation
graphs, network clustering of the proteomic data displayed similar trends with regards to fragmentation of the graphs representing the vulnerable brain regions (temporal cortex, hippocampus), whereas synaptic isolates considered resistant during ageing appear as one large network (cerebellum, occipital cortex (Fig. 29A)). Remarkably, proteomic data characterising ageing synaptic isolates from human post-mortem samples at equivalent ages to the NHP (young, mid-age, old) display strikingly similar network clustering profiles (Fig. 29B). There remains significant demarcation between the occipital cortex and the hippocampus suggesting resistant and vulnerable brain regions are ageing in unique manners in both non-human primates and human patients. Furthermore, the results also suggest that the regional NHP data may be an accurate reflection of human synaptic ageing with clear conservation of protein expression alterations during the time course.
A. Non-human primate regional ageing

Regional protein-protein correlation networks displaying proteins significantly altered through the timecourse of ageing in NHPs and human patients. Nodes signify individual proteins and edges reflect the strength of correlation of expression. Colours represent clusters of proteins that are grouped together based on their expression profiles. Correlation networks appear to be conserved in synaptosomes from human patients during normal healthy ageing. All graphs clustered using Pearson $r=0.95$.

B. Human regional ageing

To avoid ambiguity in the interpretation of complex results, further experimentation
focused primarily on a 2-way analysis. Studies documenting neuronal alterations in primates demonstrate that the occipital cortex appears to be the least affected brain region during ageing, with preservation of total neuronal numbers in NHPs (5) and volumetric preservation in aged human patients (23). Conversely, perturbations in the hippocampal architecture are often associated with advancing age due to the manifestation of Alzheimer’s disease (AD) in this region (24). Thus, there appears to be a divergent spectrum of synaptic vulnerability upon which the occipital cortex opposes the hippocampus. By utilising 2 contrasting brain regions demonstrating disparate profiles of ageing, we aimed to identify potential regulators of synaptic vulnerability with comparison of analogous protein expression profiles. Clusters displaying particular expression trends of interest were selected from both the occipital cortex and hippocampus synaptic timecourse using Biolayout Express 3D (Fig. 30A&B). Those exhibiting steady up- or down-regulation protein expression profiles during the timecourse were considered as potential biomarkers of normal healthy ageing due to the predictable age-dependent tractability of those candidates (Fig. 30A). Conversely, proteins displaying late-stage increases or decreases in expression were regarded as potential biomarkers of synaptic vulnerability as the abrupt expression changes observed in the old synaptic populations likely reflected acute alterations disrupting homeostasis at the synapse (Fig. 30B). In order to identify candidates that may be regulating regional synaptic vulnerability during ageing, proteins from occipital and hippocampal clusters with analogous expression profiles were subject to a subtractive process. Proteins demonstrating equivalent spatiotemporal profiles in both occipital cortex and hippocampal synapses were not considered to be regulators of differential regional synaptic vulnerability and filtered from the data prior to further analysis (Fig. 30C&D). Upon subtraction of proteins exhibiting analogous expression profiles, there remained 241 differentially expressed proteins that we could track through regional ageing (Fig. 31A).
Figure 30: Temporal profiling of discrete synaptic populations reveals biomarkers of ageing and vulnerability. A. Biomarkers of ageing: example temporal expression profiles of proteins demonstrating a steady up- or down-regulation during ageing. B. Biomarkers of vulnerability: example clusters displaying late stage increases or decreases in temporal protein expression. All graphs were generated in BioLayout Express\textsuperscript{3D} ($r=0.95$) and display the mean protein expression across the timecourse in occipital and hippocampal synaptic isolates. C&D. Venn diagrams indicating subtraction of candidates. Proteins demonstrating equivalent spatiotemporal profiles in both occipital cortex and hippocampal synapses (shown at intersection) were not considered to be regulators of differential regional synaptic vulnerability and were subtracted from further analysis.

5.3.4 Recapitulation of candidate protein expression promotes aberrant synaptic phenotypes \textit{in vivo}.

Although we had characterized spatiotemporal alterations occurring in populations of differentially vulnerable synapses on a global scale, it remained unclear whether the regional divergence in expression of individual candidate proteins may be capable of actively regulating synaptic vulnerability. We reasoned that candidates likely modulating alterations in the stability of the synapse, particularly at old age, would exhibit unequivocal regional temporal profiles. Proteins with corresponding regional expression at the young and mid-age time-points followed by a significant demarcation in expression at old age were selected as potential regulators of synaptic vulnerability – these particular expression profiles correlate with previous reports of significant alterations in synapse electrophysiological properties and morphometry in the aged rhesus monkey hippocampus (1, 25). With characterization of the 241 filtered proteins (Fig. 31A), we identified 8 candidates (CYC1, UQCRC1, WDR1, ROCK2, CAPZA2, OGDH, RCN2, CDH2) that displayed the archetypal regional spatiotemporal expression profile (Fig. 31B). Interestingly, all candidates demonstrated a significant reduction in expression in the old age hippocampal synapses versus the occipital cortex at the equivalent time point. To examine whether this reduction in candidate protein expression in the aged hippocampus was contributing to the vulnerability status of synaptic compartments \textit{in vivo}, we utilized a molecular genetic approach at the \textit{Drosophila} larval neuromuscular junction (NMJ)
to assess the regulatory role of individual candidates at the synapse. The larval NMJ is an excellent model system to unravel the molecular mechanisms underpinning synaptic structure, function and plasticity (26). Fundamental mammalian biological and neurological pathways demonstrate conservation in Drosophila and recent evidence has indicated that up to 75% of human disease related genes exhibit a functional ortholog in the fruit fly (27). Larval NMJs harbour glutamatergic synaptic boutons with contiguous invaginated post-synaptic membranes (sub-synaptic reticulum (SSR)) (28, 29), which display significant homology to those found in the mammalian central nervous system. Neurotransmission between these connections has the propensity to invoke plastic modifications (26, 28, 30), providing physiologically relevant mechanistic insights into proteins regulating the structure and function of the synapse in mammalian cognition (30).
Figure 31: Identification of potential candidates capable of modulating synaptic vulnerability during ageing. A. Heat map displaying average normalized abundance values of 241 differentially expressed candidates associated with synaptic vulnerability. Blue indicates low expression and red high expression. YH – young hippocampus; MH – mid-age hippocampus; OH – old hippocampus; YO – young occipital; MO – mid-age occipital; OO – old age occipital. B. Identification of proteins that may have the propensity to modulate
**Regional synaptic vulnerability.** Graphs represent temporal expression profiles of identified candidates. Note the similar expression profiles through young and mid-age in both resistant and vulnerable synaptic compartments followed by significant divergence in expression at old age. *All graphs display the ratio of candidate protein expression against the young age.* Hippocampus temporal protein expression - purple; occipital cortex temporal protein expression - grey.

Recapitulation of hippocampal candidate protein expression at the larval NMJ was achieved using the UAS/Gal4 system, promoting tissue specific expression of the selected transgenes under control of the *elav-Gal4* driver (see Table 3 for *Drosophila* orthologs). Pan-neuronal expression of the RNAi constructs (v9180; v40466; v22851; 28797; v100773; 34101; v30690; v36166) resulted in viable larva from all crosses with the exception of CAPZA2 (v100773), which demonstrated lethality at the first instar stage of development. Phenotypic assessments of muscle 12/13 NMJs in third instar larva harboring RNAi-mediated selective knock-down of single candidates revealed perturbations in multiple morphological parameters associated with the stability of the synaptic architecture (Fig. 32A-C). Bouton volume appeared significantly altered in 3 candidate lines: WDR1 (v22851), RCN2 (v30690) and CDH2 (v36166). Contrary to RCN2 and CDH2 knock-down, reductions in WDR1 protein expression demonstrated a global decrease in bouton volume (p<0.01) coupled with an increase in bouton number and NMJ arborization (Fig. 32A&B), likely due to the regulatory role of WDR1 signalling in the organization of the actin cytoskeleton (31). Conversely, selective targeting of RCN2 and CDH2 produced morphologically distinct but quantitatively similar phenotypes, with volumetric enlargement of boutons (p<0.01) alongside a reduction in bouton number. Furthermore, numerous candidate lines (WDR1, OGDH, RCN2, CDH2) demonstrated a significant loss of distinguishable type Ib (big) boutons (Fig. 32A&C) and perturbed SSR architecture (represented by DLG staining). Disruptions in SSR expression are apparent at type Is (small) boutons, with multiple candidates displaying a lack of (UQCRCl1, WDR1, RCN2), or fragmented (OGDH, CDH2), DLG staining (Fig. 32A) suggesting aberrant alterations in synaptic function. The morphological vicissitudes in multiple parameters with selective knock-down of
individual candidates suggests that these proteins may be essential to homeostatic regulation of the synaptic compartment. Thus these data represent a physiologically relevant paradigm demonstrating that reductions in the expression of these candidates in the NHP hippocampus may promote selective synaptic vulnerability during ageing.
Figure 32: Identified candidates regulate the synaptic milieu at the *Drosophila* neuromuscular junction. A. Representative confocal images of muscle 12 NMJs, labelled with anti-HRP and anti-DLG. Knock-down of candidates CYC1, UQRC1, WDR1, OGDH, RCN2 and CDH2 with RNAi demonstrates significant perturbations in the synaptic architecture. B. Graphs represent quantification of control and candidate line bouton volume (µm). WDR1 (**p = 0.0094, n=3), RCN2 (**p = 0.0033, n=3) and CDH2 (**p = 0.0024, n=3) indicate significant alterations in bouton volume. C. Graphs indicate the number of distinguishable type Ib glutamatergic boutons in control and candidate knock-down lines. WDR1 (**p = 0.0067, n=3), OGDH (**p = 0.0011, n=3), RCN2 (**p = 0.0004, n=3) and CDH2 (**p = 0.0006, n=3) demonstrate significant reductions in the number of type Ib boutons. All lines used the elav-Gal4 driver system. NMJs imaged at 63x. Scale bar = 10µm, n=8. Statistical analyses utilized unpaired two-tailed Student’s t-test (** p = <0.01; *** p = <0.001; **** p = <0.0001).

5.3.5 Selective inhibition of the TGFβ1 cascade rescues synaptic phenotypes.

Despite presenting a number of novel candidates that have the propensity to alter synaptic morphometry, it is unlikely that the expression of single proteins solely regulate regional synapse vulnerability during mammalian brain ageing. Instead, it is probable that multiple cellular and molecular pathways up- and downstream of the identified candidates converge to regulate age-dependent alterations in synaptic structure and function. By employing Ingenuity Pathway Analysis (IPA) software, we sought to identify a common upstream ‘master regulator’ that may be modulating the reduction in expression of the 8 identified candidates simultaneously. The IPA upstream analysis function highlighted that the majority of our candidates (ROCK2, CAPZA2, CYC1, CDH2, OGDH, RCN2) appeared to sit downstream of TGFβ1 (transforming growth factor beta 1) in the hierarchical cellular signalling cascade. With further examination we established that this particular pathway appeared to be activated in aged hippocampal synapses, promoting concomitant downregulation of our candidate proteins (Fig. 33). Interestingly, occipital cortex synapses of the equivalent age exhibited differential regulation of this pathway, with TGFβ1 showing significant inhibition (data not shown) and our candidate proteins...
demonstrating stable expression. Thus, there are suggestions that the activation status of TGFβ1 signalling in discrete populations of synapses may be contributing to differential synaptic vulnerability. To establish whether activation of the TGFβ1 cascade was enhancing the synaptic phenotypes described at the *Drosophila* NMJ (Fig. 32A-C), we pharmacologically manipulated expression levels with a TGFβ1 selective inhibitor (LY364947, Tocris). Inhibition of TGFβ1 with 2mM LY364947 paralleled with RNAi mediated knock-down of the candidates CDH2 and RCN2 conferred synaptic protection, with no significant differences in bouton volume detected between control and treated lines (Fig. 33A&B). In conjunction, treatment with the TGFβ1 inhibitor significantly rescued the number of type Ib glutamatergic boutons in both the RCN2 and CDH2 RNAi lines and promoted restoration of SSR architecture (Fig. 33A&C). Furthermore, NMJ arborisation appeared to be reinstated with RCN2 and CDH2 treated lines with branching and NMJ structure demonstrating morphological similitude with controls (Fig. 33A). No developmental defects were observed with LY364947 treatment and larva pupated and eclosed concurrently with corresponding control lines suggesting that there were no off-target effects. Moreover, control human patient synaptosomes demonstrated conserved reductions in RCN2 and CDH2 expression during ageing, highlighting the physiological relevance of the results described (Fig. 34). Taken together, the data demonstrate that the inherent vulnerability of aged hippocampal synapses may be mediated by perturbations in the TGFβ1 signalling cascade.
Figure 33: Pharmacological inhibition of the upstream master regulator TGFβ1 rescues synaptic phenotypes. A. Ingenuity Pathway Analysis (IPA) software highlighted TGFβ1 as a common upstream regulator to 6 candidates that have the propensity to significantly alter synaptic morphometry. The TGFβ1 signalling cascade displays significant activation in vulnerable hippocampal synaptic populations at old age promoting reductions in candidate expression. Positive z-scores indicate activation (orange) and negative z-scores indicate inhibition (blue); green represents downregulation relative to the expression in young animals. B&D. Representative confocal images of Drosophila third instar larva muscle 12 NMJs in control (left), candidate knock-down (middle) and candidate knock-
down treated with 2mM of the selective TGFβ1 inhibitor LY364947. Note the loss of stereotypic NMJ structure, bouton morphology and patterned DLG staining with both RCN2 and CDH2 RNAi lines (middle panels). RCN2 and CDH2 RNAi lines treated with 2mM LY364947 demonstrate amelioration of synaptic phenotypes (right panels). C&E. Bar charts displaying inhibition of TGFβ1 with 2mM LY364947 promotes rescue of multiple morphological parameters. Treatment revealed a significant reduction in mean bouton volume in both RCN2 (*p = 0.0244) and CDH2 (*p = 0.0274) RNAi lines versus untreated, restoring volume to control levels. In conjunction, treated lines displayed a significant increase in the number of distinguishable type Ib boutons versus untreated lines (RCN2: ***p=0.0008; CDH2: *p=0.0105). All lines used the elav-Gal4 driver system. NMJs imaged at 63x. Scale bar = 10µm, n=3. Unpaired two-tailed Student’s t-test (* p = <0.05; ** p = <0.01; *** p = <0.001; **** p = <0.0001).
Figure 34: Reductions in RCN2 and CDH2 protein expression is conserved in ageing human patient synapses. A. Actin loading control for control human patient hippocampal synaptosomes. Quantification reveals no significant difference in total protein between age groups. B. Western blot displaying RCN2 protein expression in young, mid-age and old human patient hippocampal synaptosomes. Quantification demonstrates reductions in RCN2 expression during the ageing timecourse. C. Western blot displaying CDH2 protein expression in young, mid-age and old human patient hippocampal synaptosomes.
Quantification shows a significant decrease in CDH2 expression at the old age time point. Bar charts represent average age-group protein expression quantified by fluorescence emission. YH - young hippocampus; MH – mid-age hippocampus; OH – old hippocampus. Statistical analysis was performed using an unpaired two-tailed Student’s t-test (* p = <0.05).
5.4 Discussion

The molecular mechanisms governing the age-dependent decline of selected synaptic populations remain elusive and studies are only now beginning to focus on how protein pathways may synergistically or hierarchically drive this phenomenon. Here, we demonstrate that differential synaptic vulnerability is dictated by regional divergence in protein expression. Temporal proteomic profiling of anatomically distinct brain regions from the non-human primate revealed discrete and dynamic alterations in the synaptic proteome, which appear unequivocally conserved in human patients. Utilising in silico and molecular genetic tools, we confirmed that the TGFβ1 signalling cascade demonstrates activation in aged hippocampal synapses, however the corresponding pathway appears inhibited in resistant synapses. Recapitulation of TGFβ1 activation by genetic manipulation of several downstream effector molecules demonstrated significant perturbations in the synaptic architecture in vivo. Furthermore, pharmacological inhibition of TGFβ1 ameliorated synaptic phenotypes thus affirming that enhanced transduction of TGFβ1 signalling may be modulating regional synaptic vulnerability during mammalian ageing.

5.4.1 Candidate proteins regulating age-dependent selective synaptic vulnerability.

The identification of fundamental biochemical pathways orchestrating selective synaptic vulnerability is imperative for the development of neuroprotective strategies. The current investigation suggests there is scope to detect inherent protective modulators leading to the amelioration of synaptic dysfunction. Of particular relevance are the numerous novel candidates identified, all of which have the propensity to regulate the synaptic milieu in vivo. The subcellular localisation and functional specialisation of these proteins is wide-ranging (CYC1 – mitochondria; UQCRCl – mitochondria; WDR1 – cytoplasmic/cytoskeletal; ROCK2 - cytoplasmic/cytoskeletal; CAPZA2 – cytoskeletal; OGDH – mitochondria; RCN2 – endoplasmic reticulum; CDH2 – pre- and post-synaptic membranes), likely reflecting convergence of numerous biochemical pathways required for homeostatic regulation.
of the synaptic compartment. Indeed, alterations in mitochondrial function (32), the cytoskeletal architecture (33) and calcium buffering via the endoplasmic reticulum (ER) machinery have all been widely implicated in age-dependent synaptic demise (34, 35).

Of particular interest are the candidates reticulocalbin 2 (RCN2) and cadherin 2 (CDH2), as upstream pharmacological intervention promoted significant amelioration of the associated synaptic phenotypes at the \textit{Drosophila} NMJ. RCN2 is a known EF-hand calcium-binding protein (36) with strict localisation to the ER-lumen (37) and has previously been associated with numerous neurological diseases, including absence epilepsy (38) and multiple sclerosis (39). In addition, fibroblasts derived from elderly human patients have reported significant reductions in RCN2 expression suggesting the protein may be relevant to the cellular alterations underpinning senescence (40). Furthermore, recent experimental evidence has demonstrated that RCN2 directly interacts with the mitochondrial sideroflexin (SFXN) protein family in human patients (41), which we have shown to be α-synuclein dependent regulators of the synaptic architecture \textit{in vivo} (42). Taken together, the data indicate that this particular protein may play a role in mediating age-related synaptic demise via disruptions in calcium-dependent signalling at the ER-mitochondrial axis. Further investigations into RCN2 signal transduction and mitochondrial effector molecules may aid in determining the pathophysiological role of the ER and its associated mitochondrial complexes and how these may modulate synaptic stability during ageing and disease.

In addition to RCN2, we also demonstrated phenotypic rescue of the CDH2 misexpression \textit{Drosophila} line. CDH2, or N-cadherin, is a cell adhesion molecule, acting as synaptic ‘glue’ to ensure appropriate recognition and connectivity of the pre- and post-synaptic membranes (43, 44). The hippocampus exhibits clustering of CDH2 protein within the active zones of excitatory synapses (45) and appears to modulate synaptic plasticity, synaptic vesicle docking and recycling, post-synaptic density stabilisation and dendritic spine morphology \textit{in vitro} (46). Interestingly, expression of CDH2 is required for the induction of long-term potentiation (LTP)
suggesting homeostatic control of the protein is essential to both the structural integrity and functional activity of the synapse (47). Indeed, reductions in neuronal CDH2 expression promoted aberrant pre- and post-synaptic organisation at the *Drosophila* NMJ suggesting perturbed synaptic functions via impairments in activity-dependent morphological remodelling - processes which are believed to widely contribute to age-related cognitive decline.

5.4.2 The TGFβ1 signalling cascade: a synaptic-microglial intersection?

Despite the 8 candidates displaying significant diversity in localisation and function at the synapse, surprisingly, 6 proteins demonstrated a common upstream regulator: TGFβ1. Intriguingly, TGFβ1 is not constitutively expressed in neurons, but displays robust levels of expression in microglia (Fig. 35A) suggesting that regional synaptic vulnerability may be mediated by microglial signalling. Indeed, recent investigations have reported age- and region-dependent microglial diversity at the transcriptome level, with indications that hippocampal microglia exhibit compromised function with advancing age (48). In conjunction, immunohistochemical techniques have revealed that microglial populations in aged NHP models and human patients appear to display a dystrophic morphology (49). In conjunction with this senescent phenotype, there is abundant evidence to suggest that microglia become hypersensitive or ‘primed’ resulting in prolonged pro-inflammatory activation in response to homeostatic alterations (50, 51), which may functionally contribute to age-related neuronal alterations via modifications in cytokine signalling and immuno-surveillance (52). Furthermore, investigations into the pathogenic role of microglia have documented chronic over-expression of TGFβ1 in multiple neurodegenerative diseases, including Alzheimer’s disease (AD), frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS) and Parkinson’s disease (PD) (53, 54), illustrating that this pathway may be contributing to numerous age-related diseases in which the synapse is an early pathological target.
Figure 35: Regional synaptic vulnerability is regulated by the microglia. A. Bar chart displaying TGFβ1 transcript expression in isolated hippocampal microglia and hippocampal homogenate. The homogenate exhibits relatively little TGFβ1 expression versus the isolated microglia (unpaired two-tailed student’s t-tests, p = 0.0009). B&C. Summary of synaptic-microglial interactions and regulation of the cellular milieu during ageing. Young adult microglia (B) demonstrate ramified morphology and inhibition of the TGFβ1 signalling cascade, promoting homeostatic regulation of the subcellular machinery within the synaptic compartment. Intracellular processes are illustrated in the black box. Conversely, resident microglia in an aged brain (C) display a dystrophic, primed phenotype. Activation of the TGFβ1 signalling cascade, by binding of TGFβ1 to the TGFβ1 synaptic receptor, promotes injurious events within the synaptic terminal facilitating synapse instability. Orange colour represents dendritic arbour with synaptic terminals; pale blue indicates microglia; white synaptic terminals symbolises dysfunction. See key in black box for intracellular information.

Based upon this evidence, it is perhaps unsurprising that our data have described microglial TGFβ1 signalling as a fundamental modulator of regional synaptic vulnerability during ageing. Of course, microglia and synapses are not mutually exclusive entities and there is a requirement for a dynamic and bi-directional relationship to enable homeostatic control of the cellular milieu however, this critical intersection appears to be potentiating age-dependent instability in the hippocampal synaptic architecture. Despite the wealth of data presented in this study, the cause-
consequence relationship underpinning selective synaptic vulnerability remains unresolved. It is unclear whether i) disruptions in synaptic proteostasis precede aberrant microglial TGFβ1 signalling or ii) microglia are the primary effectors and activation of TGFβ1 signalling facilitates synaptic dysfunction. However, we may speculate that the latter appears to be the most compelling hypothesis as restoration of upstream microglial signalling promotes a full rescue of the synapse in multiple models of synaptic ageing (Fig. 35B&C). Correspondingly, supporting our proposed hypothesis are the pioneering heterochronic parabiosis studies by Villeda et al, 2011 (55), who describe TGFβ1 as a pro-ageing cytokine underpinning hippocampal-dependent cognition. In agreement with the current study, experimental procedures inhibiting TGFβ1 (and other microglial-regulated immunomodulatory factors) rescued neurogenesis and concomitant learning deficits in aged mice. Thus, the microglial-synaptic axis appears to be a fundamental intersection that requires a fine-tuned equilibrium to maintain selective synaptic integrity during advancing age.

Cumulatively, our data provide a comprehensive insight into the regional heterogeneity of synaptic ageing and how proteostatic alterations may dictate selective synaptic vulnerability in the primate. Identification of the microglial cytokine TGFβ1 as a master regulator of hippocampal synapse structure and function is indicative of a highly dynamic cohesive cellular network that requires symbiotic modulation for optimal neuronal function during advancing age. Despite this, the implicit role of the TGFβ1 cascade in regional synaptic vulnerability is not unequivocal. It remains unclear whether activation of TGFβ1 signalling is uniquely maladaptive to selected synaptic populations or whether discrete target tissues demonstrate varied allostatic loads. Furthermore, a significant chasm in scientific knowledge concerning microglial-synaptic interactions during advancing age persists and this must be addressed before we can fully appreciate the functional pathophysiology of age-related cognitive decline.
Chapter 6. Proteomic profiling of differentially vulnerable synaptic populations in the ageing human patient

6.1 Introduction

Despite erudite research efforts many of the causative mechanisms driving human neurodegenerative disease manifestation and progression remain to be elucidated. It has been well established that advancing age is the single largest risk factor for the development and diagnosis of a neurodegenerative disease, with Alzheimer’s disease (AD) being the most prevalent condition (251). AD has a destructive impact on the patient’s capacity to function independently paralleled with significant cognitive and behavioural alterations (2, 3) reflecting the gross pathological alterations occurring within the intricate cortical structures of the brain (116). Due to the prevalence, severity and devastating consequences of AD, research focus has been directed towards unravelling the causative mechanisms of the disease; however, there has been relatively little emphasis on the correlates of normal healthy brain ageing in the human patient and the impact that advancing age has on the cortical cellular architecture in the absence of pathogenic processes. It has become increasingly evident that normal ageing has the propensity to promote progressive cognitive impairment with a multitude of aged individuals exhibiting selective reductions in executive function, short-term memory capacity and retention of novel information (3-5). The clinical symptomology described is common to both the very early stages of AD and advancing age suggesting age-related cognitive impairment and the early pathologic processes occurring in AD may demonstrate a convergence of analogous biochemical cascades. To appreciate the complex intersection of human advancing age and AD pathogenesis, it is essential that the field characterises neuronal biochemical alterations that may be regarded as ‘normal’ and those that may act as pathogenic precursors to the development of neurodegenerative diseases. Without a comprehensive understanding of the perturbed pathways governing age-related alterations in the neuronal milieu, there are limited possibilities for the development of successful strategies that promote cognitive health in the human patient.
As outlined in Chapters 1&5, synapses are particularly vulnerable during advancing age and discrete neuroanatomical regions demonstrate selective susceptibility to insult. It is well established that the hippocampal synaptic architecture displays particular vulnerability to a wide range of insults during advancing age (4, 5, 93), whereas synapses resident to the occipital cortex exhibit resistance to age-related alterations (3, 94, 116-121, 224, 225). Despite this, there are relatively few studies investigating the molecular correlates bolstering selective synaptic vulnerability, particularly in human tissues.

Here, in what appears to be the first investigation exploring the temporal molecular alterations occurring in neuroanatomically distinct populations of synapses during the human lifespan, we describe data supporting the notion that local alterations in the regional synaptic proteome may facilitate synaptic vulnerability during ageing. Comparative proteomic profiling of hippocampal and occipital cortex synapses revealed regional divergence in protein expression, promoting the identification of 25 novel candidates that may be associated with synaptic vulnerability during advancing age in the human patient. A number of the identified candidates clustered into the TGFβ1 hierarchical signalling cascade, which exhibited differential activation in the hippocampus and occipital cortex at old age. The data support our previous observations in Chapter 5 and suggest that the activation status of the TGFβ1 pathway may mediate the age-dependent vulnerability of hippocampal synapses in human patients.
6.2 Methods

6.2.1 Human patient samples
Human patient samples were obtained from the Edinburgh Brain Bank. All tissues were classified as controls due to the absence of gross pathological hallmarks and neurological disease. Human tissues were assigned to equivalent age groups: young (18-25 years), mid-age (40-50 years) or old age (70+ years), with 4 samples per time point. Use of human tissue for post-mortem studies was reviewed and approved by the Edinburgh Brain Bank ethics committee. The Edinburgh Brain Bank is a Medical Research Council funded facility with research ethics committee approval (11/ES/0022).

6.2.2 Synaptosomal preparations
Regional synaptosomal preparations were performed on the human patient young, mid-age and old hippocampal and occipital cortex samples using the methods described in Chapter 2, section 2.3.

6.2.3 Protein concentration assay
Protein concentration of individual patient samples and pooled regional samples was determined utilizing the methods described in Chapter 2, section 2.5.

6.2.4 Label-free proteomics
Human patient regional synaptosomal preparations were extracted in SDT lysis buffer containing 100 mM Tris-HCl (pH 7.6) and 4% (W/V) sodium dodecyl sulfate (VWR). For efficient protein extraction, lysates were freeze–thawed and homogenized in SDT buffer several times. Protein concentrations were then determined using BCA assay.

The following mass spectrometry analyses were performed by the FingerPrints proteomic facility at the University of Dundee. Aliquots (200 µg) of each synaptosomal preparation were processed through FASP (filter-aided sample preparation) involving buffer exchange to 8 M urea and alkylation with 50 mM
iodoacetamide prior to a double digestion with trypsin (Roche, sequencing grade), initially for 4 h, then overnight at 30 °C. Trypsin-digested peptides were separated using an Ultimate 3000 RSLC (Thermo Scientific) nanoflow LC system with the column oven set to 35 °C. Technical replicates (3 × ~1 µg) of each sample were loaded at a constant flow of 5 µL/min onto a trapping cartridge (PepMap100, C18, 5µm, 100Å 0.3 x 5 mm; (Thermo Scientific, San Jose, CA) using 2% Acetonitrile, 0.1 % formic acid. After trap enrichment, peptides were separated on a peptide CSH, 1.7µm, 130Å, 75µm x 250mm C18 column (Waters Corp, Milford, MA) with the following gradient: t=0 min, 2 % B; t=6, 2 % B; t=20, 8 % B; t=110, 24 % B; t=135, 37 % B where solvent A is water with 0.1 % formic acid and solvent B is 80% acetonitrile with 0.1% formic acid, with a constant flow of 260 nL/min. The HPLC system was coupled to a linear ion trap Orbitrap hybrid mass spectrometer (LTQ-Orbitrap Velos Pro, Thermo Scientific) via a nanoelectrospray ion source (Thermo Scientific). The spray voltage was set to 2.2 kV, and the temperature of the heated capillary was set to 200 °C. Full-scan MS survey spectra (m/z 335–1800) in profile mode were acquired in the Orbitrap with a resolution of 60 000 after accumulation of 1 000 000 ions. A lock mass of 445.120 024 was enabled for survey scans to improve mass accuracy. The 15 most intense peptide ions from the preview scan in the Orbitrap were fragmented by collision-induced dissociation (normalized collision energy, 35%; activation Q, 0.250; and activation time, 10 ms) in the LTQ after the accumulation of 10 000 ions. Dynamic exclusion parameters were set as follows: repeat count, 1; repeat duration, 30 s; exclusion list size, 500; exclusion duration, 45 s; exclusion mass width, plus/minus 10 ppm (relative to reference mass). Maximal filling times were 10 ms for the full scans and 100 ms for the MS/MS scans. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged species were rejected. Data were acquired using Xcalibur software.

The following analyses described were performed by Laura Graham at the Roslin Institute. Raw proteomic data were imported into Progenesis for characterization and analysis of relative ion abundance. 2D representations of MS/MS output were created for each sample and these were aligned to determine similar features.
(average alignment score >88%). Following alignment, data was filtered by retention time with features detected below 11.81 minutes and above 134.37 minutes discarded to correct for elution variability. The runs were grouped according to age and brain region and Statistical P values were automatically generated in Progenesis software through a 1 way ANOVA on the ArcSinh transform of the normalized data.

Peptides were filtered by the following criteria: power <0.8, fold change >1.2, p>0.05 and the remaining data were exported from Progenesis for identification of individual peptide sequences using the IPI-\textit{homo sapiens} database via Mascot Search Engine (V2.3.2). Enzyme specificity was set to that of trypsin, allowing for cleavage N-terminal to proline residues and between aspartic acid and proline residues. Other parameters used were as follows. (i) Variable modifications: methionine oxidation, methionine dioxidation, protein N-acetylation, gln \rightarrow pyro-glu. (ii) Fixed modifications: cysteine carbamidomethylation. (iii) MS/MS tolerance: FTMS- 10 ppm, ITMS- 0.6 Da. (iv) Minimum peptide length: 6. (v) Maximum missed cleavages: 2. (vi) False discovery rate: 1%. A cutoff score of >34 was used based on Mascot probability threshold of 0.05 that the observed hit is a random event. As an indication of identification certainty, the false discovery rate for peptide matches above identity threshold was set at 1%.

Identified proteins were re-imported into Progenesis for further processing. Proteins were subject to stringent filtering parameters to eliminate those which had <2 unique peptides, <1.2 fold change between age groups and p>0.05 to obtain the proteins which demonstrated the largest significant variation in expression over the ageing time course in each brain region.

\textbf{6.2.5 Biolayout Express$^{3D}$}

Proteomic data was dissected using the complex pattern recognition software, Biolayout Express$^{3D}$ (159). The software allows visualization of molecular networks by applying Markov clustering algorithms to raw proteomic data (MCL 2.2). All graphs were clustered using Pearson correlation r=0.97. Clusters of interest indicating age-dependent alterations included those that demonstrated a steady up- or
down-regulation or a late stage up- or late stage down-regulation during the timecourse of ageing. Proteins from clusters with analogous expression profiles underwent a subtractive process – candidates appearing in both the resistant and vulnerable clusters, altered in the same manner, were unlikely to regulate synaptic vulnerability during ageing. These candidates were eliminated as modulators of synaptic vulnerability.

6.2.6 Ingenuity Pathway Analysis

IPA analyses were performed as previously described (157). See methods section 2.9 for details on the parameters utilized for experimental analysis.

6.2.7 Quantitative Fluorescent Western Blotting

See Chapter 2, section 2.10 for a detailed methodological overview. All antibodies utilised are listed in Tables 1 & 2.
6.3 Results

6.3.1 Temporal proteomic profiling of anatomically distinct synaptic isolates from the human patient brain.

As highlighted in chapters 1 and 5, discrete neuroanatomical brain regions harbour differential vulnerabilities to insult and degeneration during advancing age. Despite concerted efforts to characterise the age-dependent patterned morphological alterations occurring in neuronal subpopulations, few investigations have examined the temporal biochemical cascades that may facilitate susceptibility or resistance to injurious stimuli in the human patient. To investigate the regional temporal molecular alterations occurring in human synapses during normal healthy ageing, we purified and characterised crude synaptosomes from two differentially vulnerable brain regions (occipital cortex and hippocampus) at 3 time points (young adult, mid-age, old). Quantitative label-free proteomic analyses identified >1570 proteins in both regions across the time-course, revealing dynamic divergences in synaptic protein expression. Remarkably, over 1000 proteins were altered by greater than 20% in both the hippocampus and occipital cortex, demonstrating the significant age-dependent biochemical adaptations occurring in discrete synaptic populations (Fig. 36A&B). Purity of regional synaptic preparations was assessed utilising PANTHER Gene Ontology bioinformatics software. Input of the 1574 identified proteins from the proteomic data indicated significant enrichment of postsynaptic membranes suggesting relative purity of the synaptosomal preparations (Fig. 36C). Additionally, to determine the veracity of the proteomic data we employed quantitative fluorescent western blotting. Corresponding protein expression trends were observed in the human tissue preparations as indicated by the proteomics for the proteins citrate synthase (Fig. 37C&D) and ROCK2 (Fig. 37E&F). Collectively, the data indicate the relative purity of the synaptic preparations and suggest the proteomic data is representative of the molecular alterations occurring in the human synapses during ageing.
Figure 36: Spatiotemporal characterisation of regional synaptic proteomes in the human patient. A&B. Venn diagrams demonstrating regional characterization of the synaptic proteome. Proteins were identified and filtered in Progenesis using the following criteria: \( p > 0.05 \), \(< 1.2 \) fold change across the timecourse and 1 unique peptide to obtain the proteins that demonstrate the largest alterations during normal healthy ageing in human patients. Number of proteins up- or down-regulated by \( >1.2 \) fold change during ageing, is indicated at the middle intersection. These filtered proteins were utilized for all analyses. C. PANTHER GO-Slim cellular component enrichment analysis of the temporal hippocampal and occipital cortex proteomic data. Table indicates enrichment of the postsynaptic membrane suggesting relative purity of preparations. *Fold-enrichment values greater than 1 denote overrepresentation of category in dataset.*
Figure 37: Validation of human regional synaptic alterations with quantitative fluorescent western blotting. A. βIII-tubulin loading control demonstrating equal loading between individual patients and between regions. B. Graph displays no significant difference in βIII-tubulin expression between pooled samples. C-F. Upper bar charts display the proteomic average normalised expression values of the proteins citrate synthase (C.) and ROCK2 (E.) in regional synapses during ageing. Lower bar charts demonstrate sample protein expression quantified by the fluorescent western blots shown. Proteomic and sample expression of both citrate synthase (D.) and ROCK2 (F.) follow the same trend thereby providing validation of the proteomic data. Y1-3 = young adult individual human patient synaptic samples; M1-3 = mid-age adult individual human patient synaptic samples; O1-3 = old adult individual human patient synaptic samples. YH = young patient pooled hippocampal synapses; MH = mid-age patient pooled hippocampal synapses; OH = old patient pooled hippocampal synapses; YO = young patient pooled occipital cortex synapses; MO = mid-age pooled occipital cortex synapses; OO = old patient pooled occipital cortex synapses. Statistical analysis was performed using an unpaired two-tailed Student’s t-test.

6.3.2 Regional heterogeneity of the human synaptic proteome during normal healthy ageing

To examine whether synapses from differentially vulnerable brain regions aged in a similar manner, we utilized the objective network visualisation software Biolayout Express3D (www.biolayout.org) to generate brain regional principal component analysis correlation graphs demonstrating relative age-dependent similarities (Fig.
Examination of these networks verified the presence of distinct region-dependent clustering profiles. Interestingly, the graph representing the temporal timecourse of hippocampal synaptic ageing demonstrates division into 2 discrete networks, indicating age-dependent heterogeneity. Fragmentation suggests that the aged hippocampal synapses possess discrete protein expression profiles (Fig. 38A) versus the young and mid-age synaptic isolates, which may contribute to the vulnerability status of synaptic compartments at this particular age. Though the resistant occipital cortex synapses also demonstrate a fragmented profile (Fig. 38B), it appears that mid-age synaptic isolates exhibit reduced equivalence with the young and old time points. The salient similarities in protein expression between the young and old occipital cortex synaptic populations may confer resistance to insult during advancing age. The data provide indications that isolated human synaptic populations age in a region-dependent manner and these unique alterations may dictate potential vulnerability of synapses during advancing age.

**Figure 38:** Human synaptic ageing is regionally heterogeneous. Unbiased sample-sample correlation analysis generated from BioLayout Express³D software. Nodes signify individual samples and edges reflect the strength of correlation of expression. YH = young patient pooled hippocampal synapses; MH = mid-age patient pooled hippocampal synapses; OH = old patient pooled hippocampal synapses; YO = young patient pooled occipital cortex synapses; MO = mid-age pooled occipital cortex synapses; OO = old patient pooled occipital cortex synapses. All graphs clustered using Pearson r=0.98.
6.3.3 Regional profiling of hippocampal and occipital cortex synapses reveals protein expression profiles associated with synaptic vulnerability

In conjunction with the principal component analyses, to dissect region-specific age-dependent synaptic alterations further, we generated network graphs of the regional timecourse proteomic data, again utilising Biolayout Express3D (159). The software applies unbiased Markov clustering algorithms to the input data and groups proteins displaying similar expression trends. This allows visualisation of spatiotemporal profiles promoting the identification of physiological cascades altered within the dataset. Graphs were constructed utilizing regional differentially expressed proteins (altered >20%) through the timecourse of ageing (see Fig. 36A&B) providing 40-50 clusters per region. Interestingly, in contrast to the principal component analyses, both the hippocampal and occipital cortex timecourse data exhibit one large interconnected network (Fig. 39), though there is a marked increase in fragmentation in the hippocampal synapses (Fig. 39A). This appears to be in agreement with the principal component analysis correlation graphs and is likely reflective of the heterogeneity in protein expression between the young, mid-age and old hippocampal synaptic populations (see Fig. 38A). Conversely, the occipital cortex timecourse displays a single network with little fragmentation (Fig. 39B), suggesting that a multitude of proteins may harbour similar protein expression profiles despite the reported heterogeneity between age groups (Fig. 38B). Although the networks display subtle demarcations between hippocampal and occipital cortex temporal synaptic protein expression, the data indicate that resistant and vulnerable brain regions may be ageing heterogeneously in human patients.
Figure 39: Vulnerable and resistant synaptic populations exhibit subtle alterations in protein clustering. Regional protein-protein correlation networks displaying proteins significantly altered through the timecourse of ageing in human patient hippocampal and occipital cortex synapses. Nodes signify individual proteins and edges reflect the strength of correlation of expression. Colours represent clusters of proteins that are grouped together based on their expression profiles. All graphs clustered using Pearson r=0.97.

As highlighted in chapter 5, there appears to be a divergent spectrum of synaptic vulnerability upon which the occipital cortex opposes the hippocampus. By utilising 2 contrasting brain regions demonstrating disparate profiles of ageing, we aimed to identify potential regulators of synaptic vulnerability with comparison of analogous protein expression profiles. Clusters displaying particular expression trends of interest were selected from both the occipital cortex and hippocampus synaptic timecourse using Biolayout Express3D (Fig. 40A&B). Those exhibiting steady up- or down-regulation protein expression profiles during the timecourse were considered as potential biomarkers of normal healthy ageing due to the predictable age-dependent tractability of those candidates (Fig. 40A). Conversely, proteins displaying late-stage increases or decreases in expression were regarded as potential
biomarkers of synaptic vulnerability as the abrupt expression changes observed in
the old synaptic populations likely reflected acute alterations disrupting homeostasis
at the synapse (Fig. 40B). In order to identify candidates that may be regulating
regional synaptic vulnerability during ageing, proteins from occipital and
hippocampal clusters with analogous expression profiles were subject to a
subtractive process. Proteins demonstrating equivalent spatiotemporal profiles in
both occipital cortex and hippocampal synapses were not considered to be regulators
of differential regional synaptic vulnerability and filtered from the data prior to
further analysis (Fig. 40C&D). Upon subtraction of proteins exhibiting analogous
expression profiles, there remained 583 differentially expressed proteins that we
could track through regional ageing.
Figure 40: Temporal profiling of discrete synaptic populations reveals biomarkers of human synaptic ageing and vulnerability. A. Biomarkers of ageing: example temporal expression profiles of proteins demonstrating a steady up- or down-regulation during ageing. B. Biomarkers of vulnerability: example clusters displaying late stage increases or decreases in temporal protein expression. All graphs were generated in BioLayout Express$^{3D}$ ($r=0.97$) and display the mean protein expression across the timecourse in occipital and hippocampal synaptic isolates. C&D. Venn diagrams indicating subtraction of candidates. Proteins demonstrating equivalent spatiotemporal profiles in both occipital cortex and hippocampal synapses (shown at intersection) were not considered to be regulators of differential regional synaptic vulnerability and were subtracted from further analysis.

6.3.4 Activation of the TGFβ1 signalling cascade modulates candidates associated with synaptic vulnerability

Despite characterizing the global spatiotemporal changes occurring in differentially vulnerable synaptic populations, it remained unclear whether divergence in expression of individual candidate proteins may be capable of actively regulating synaptic vulnerability. To elucidate regional regulators of synaptic vulnerability during advancing age, we initially mapped the individual temporal expression profiles of the 583 candidates identified by the BioLayout Express$^{3D}$ analyses using Python Jupyter Notebook (see Appendix 4). We sought to identify candidate proteins demonstrating corresponding regional expression at the young and mid-age time-points followed by a significant demarcation in expression at old age – these particular candidates were considered potential regulators of synaptic vulnerability due to the reasoning outlined in Chapter 5, section 5.3.4. Additionally, we aimed to replicate the methodological parameters utilized for the analysis in Chapter 5 to facilitate direct comparisons of the ageing NHP and human regional timecourse data and delineate whether similar candidates may be modulating regional synaptic ageing between species. Upon tracking of the 583 candidates, we identified 25 proteins that displayed the archetypal spatiotemporal expression profile (Fig. 41). These candidates represented potential regulators of regional age-dependent human synaptic vulnerability.
Figure 41: Candidate proteins associated with regional differential synaptic vulnerability during advancing age. Graphs display the temporal expression profiles of the 25 candidates identified by Biolayout Express3D analyses. Data is displayed as a ratio of candidate protein expression against the young age. Note the similar expression profiles through young and mid-age in both resistant and vulnerable synaptic compartments followed by significant divergence in expression at old age. Hippocampal temporal protein expression - blue; occipital cortex temporal protein expression – green; red lines indicate 20% change. Graphs constructed using Python Jupyter Notebook.

Though the 25 candidates did not include those identified in Chapter 5, by employing Ingenuity Pathway Analysis (IPA) software, we sought to identify whether the aged NHP and human hippocampal synapses harboured a common upstream regulator that may be globally modulating the expression of proteins associated with increased synaptic vulnerability (Fig. 31&41). Indeed, the IPA upstream analysis function highlighted that 11 of our candidates identified from the
human data (Fig. 41) also sit downstream of TGFβ1 in the hierarchical cellular signalling cascade (Fig. 42), and, similar to the NHP data, this particular pathway appeared to be activated in aged hippocampal synapses (Fig. 42A). Interestingly, occipital cortex synapses of the equivalent age exhibited differential regulation of this pathway, with TGFβ1 showing significant inhibition (Fig. 42B). Activation status of the TGFβ1 signalling cascade appears to mediate the regional expression of selected protein candidates (APOA1, SLC7A5, CDC42EP4, IGKC, CD99, STMN3, HCN2, PPT1, FKBP4, HEBP1, PDAP1) in aged humans suggesting that TGFβ1 may be an important biochemical substrate contributing to differential synaptic vulnerability during advancing age (please refer to Chapter 5 for supporting in vivo data).
Figure 42: Activation status of the TGFβ1 signalling cascade modulates differential synaptic vulnerability in the aged human patient. A. The TGFβ1 signalling cascade displays significant activation in vulnerable hippocampal synaptic populations at old age promoting concomitant alterations in downstream candidate expression. The candidates APOA1, SLC7A5, CDC42EP4, IGKC, CD99, STMN3 are displayed at the bottom of the cascade. B. TGFβ1 signalling demonstrates significant inhibition in resistant occipital cortex synapses at old age, which modulates the expression of selected candidates downstream. The candidates HCN2, PPT1, FKBP4, HEBP1, PDAP1 are displayed at the bottom of the network. Positive z-scores indicate activation (orange) and negative z-scores indicate inhibition (blue); green represents downregulation relative to the expression in young patients; red represents upregulation relative to protein expression in young patients.
6.4 Discussion

The molecular mechanisms governing the selective vulnerability of discrete synaptic populations in the human patient during advancing age remain unresolved. In what appears to be the first investigation exploring the molecular alterations occurring in neuroanatomically distinct populations of synapses during the human lifespan, we describe data supporting the notion that local alterations in the regional synaptic proteome may facilitate synaptic vulnerability during aging. Comparative proteomic profiling of hippocampal (age-vulnerable) and occipital cortex (age-resistant) synapses demonstrated regional divergence in protein expression, revealing novel candidates that may be capable of actively regulating synaptic stability during advancing age in the human patient. A number of the identified candidates clustered into the TGFβ1 hierarchical signaling cascade, which exhibited differential activation in the hippocampus and occipital cortex at old age. The data support our previous observations in Chapter 5 and suggest that the activation status of the TGFβ1 pathway may mediate the age-dependent vulnerability of hippocampal synapses in human patients as well as rhesus macaques.

Though we have identified similar activation of the TGFβ1 hierarchical signaling cascade to the NHP in both the aged human hippocampus and occipital cortex, we were unable to determine whether experimental manipulation of the 11 candidates associated with this pathway promoted aberrant synaptic phenotypes in vivo. However, previous studies have established that misexpression of APOA1, SLC7A5, CDC42EP4, IGKC and CD99 correlate with perturbations in the synaptic-microglial axis and may facilitate pro-inflammatory states, increasing the risk of AD or dementia development (252-258). Thus, it is likely that TGFβ1 is an important modulator of selective synaptic vulnerability due to conserved regional activation of the pathway across multiple primate species during advancing age and the reported downstream candidate associations with synaptic dysfunction. Although discussed in length in Chapter 5, section 5.4.2, holistic investigations exploring the effect of TGFβ1 expression on synaptic structure, function and cognitive status during normal healthy aging may provide increased mechanistic insights into the biochemical
substrates orchestrating selective synaptic vulnerability and enable identification of appropriate modulatory candidates for therapeutic intervention. Elucidation of such mechanisms will be imperative for the development of strategies promoting cognitive health in human patients.

The use of human patient tissue is fundamental for the extrication of the molecular mediators promoting age-related cognitive decline and neurodegenerative diseases. Though a precious and enlightening resource, significant challenges present when utilising human samples, particularly in biochemical studies. Investigations examining post-mortem preservation of the cortical molecular milieu describe several interfering variables that appear to affect protein integrity, expression and post-translational modifications, including cause of death, duration between death and cadaver refrigeration – as well as refrigeration temperature and post-mortem interval (259, 260). The post-mortem interval is typically reported in studies utilising human tissue and unless significantly extended (>50 hours) accounts for relatively little degradation at the protein level, providing tissue has been preserved and stored correctly (260). However, the time between death and refrigeration of the body has been demonstrated to have a profound impact on protein integrity, with marked degradation occurring at temperatures >1°C (259). Though we report patient post-mortem intervals of <50 hours, and appropriate tissue storage at -80°C, the duration period between death and refrigeration remains elusive. Whilst we attempted to dilute sample-sample variability by pooling human patient tissues by age and region, uncontrolled post-mortem factors may have impacted upon the quality of the proteomic data presented.

Although inter-patient variability may be substantial due to epigenetic modifications facilitated by dietary, health and environmental factors, in addition to the post-mortem aspects described above, it is incredibly significant to the trajectory of ageing and cognitive capacity. It is well established that only selected individuals develop age-related cognitive decline thus assessing the variability in biochemical profiles between patients may enable the elucidation of biomarkers relevant to the manifestation of synaptic dysfunction and concomitant cognitive impairment. Whilst
we describe a study assessing the global alterations commonly occurring in pooled human regional synaptic populations, investigations delineating how the differential expression of discrete molecular substrates between patients may modulate cognitive capacity are fundamental to our understanding of selective synaptic dysfunction during advancing age.

Despite the caveats, we describe a novel dataset documenting the age- and region-dependent alterations occurring in the human patient brain. This comprehensive comparative proteomic profiling has provided unique insights into how differential synaptic vulnerability may be modulated by the TGFβ1 signalling cascade, which may have wide implications for the field of neurodegenerative diseases. Furthermore, the generation of a dynamic temporal synaptic map provides an important resource that we may further compare disease processes to, allowing elucidation of biochemical alterations that may be regarded as pathogenic.
Chapter 7. General discussion

7.1 Overview of results
The data presented in this thesis provide a comprehensive insight into the dynamic spatiotemporal regulation of the synaptic compartment during normal healthy ageing. Utilising a methodological workflow developed for such analyses, we describe several novel comparative studies documenting the biochemical alterations occurring in both discrete mitochondrial and synaptic populations during advancing age. Though our initial investigation focused on the mitochondrial molecular milieu at a static time point, we demonstrate that mitochondria harbour discrete proteomes dependent upon subcellular localisation. Additionally, recapitulation of the synaptic mitochondrial proteome has the propensity to promote perturbations in the presynaptic terminal, suggesting that the dynamic divergence in subcellular mitochondrial protein expression may modulate the stability of the synapse. Indeed, with spatiotemporal characterisation of the rodent synaptic and non-synaptic mitochondrial proteomes, we report significant age- and compartmental-specific alterations in protein expression, which appear to mediate the structural and functional properties of the synapse during ageing. Together these data indicate that proteostatic regulation of the synaptic mitochondrial proteome is vital for the sustained architectural integrity of the cortical synapse during advancing age.

In addition to examining the temporal biochemical regulation of isolated organellar subpopulations, we also characterised the global molecular changes occurring in differentially vulnerable synaptic populations in the NHP and human patient during normal healthy ageing. Comparative proteomic profiling of hippocampal and occipital cortical synapses revealed novel modulators of synaptic stability as well as mechanistic pathways regulating the selective vulnerability of discrete synaptic populations. We demonstrate that expression of the upstream regulator TGFβ1 modulates synaptic vulnerability and pharmacological manipulation of the protein ameliorates age-associated synaptic phenotypes. Remarkably, TGFβ1 appears to be a conserved regulator of regional synaptic stability in both the ageing NHP and human
patient, suggesting that the candidate may warrant further investigation as a potential therapeutic target for age-related cognitive decline.

Collectively, this thesis provides the first molecular insights into differential proteostatic regulation of the synaptic compartment during ageing and how discrete alterations in protein expression may dictate vulnerability. Elucidation of the TGFβ1 signalling cascade as a conserved mechanistic regulator of differential synaptic vulnerability during advancing age provides insights into the dynamic age-dependent modulation of the synaptic-microglial axis, which may be of paramount importance with regards to preservation of the synaptic milieu. Furthermore, the generation of species-specific temporal synaptic fingerprints provides a novel and essential resource that we may further compare disease processes to, allowing elucidation of biochemical alterations that may be regarded as pathogenic.

### 7.2 Rodents: a representative model of human ageing?

Although we have utilised rodents as a convenient experimental model of synaptic ageing in Chapters 3&4, recent reports have indicated that the temporal regulation of gene expression in the mouse cortex during advancing age is not well conserved in higher order mammals (138). Indeed, with comparative protein profiling of the mouse, rhesus macaque and human synaptic ageing datasets presented in this thesis (Chapters 4, 5 & 6), we demonstrate significant divergence in temporal protein expression in mouse cortical synapses during advancing age (Fig. 43), suggesting that rodent models may not faithfully recapitulate the dynamic biochemical alterations occurring in primate synapses during ageing. Indeed, with comparison of rhesus macaque and human synaptic ageing, we reveal significant similitude in temporal protein expression (Fig. 43), indicating that conserved regulation of the synaptic proteome may be unique to primate species. It appears likely that the differential biochemical regulation of the primate synaptic milieu is due to evolutionary divergence of the cortex as opposed to species-specific differences in maximal lifespan, as ageing of NHP and human synapses indicate analogous temporal protein expression alterations despite significantly varied biological ages.
Though the evidence appears to support the notion that ageing primate synapses may demonstrate discrete modulatory mechanisms during advancing age, some discrepancies between the primate and rodent datasets may also exist due to the use of varied populations of synapses and biochemical isolation methods (please refer to Chapters 4, 5 and 6 for methodological details). Although the modifications in methodological parameters between the rodent, NHP and human patient datasets is an important consideration, the results described correlate with previously reported cross-species transcriptomic ageing data (138), suggesting that this analysis may be a reliable representation of species-specific synaptic protein expression during advancing age.

Accordingly, the preferential use of rodents in brain ageing and neurodegenerative disease studies may be hindering the detection of viable and translatable functional mediators that facilitate synaptic and/or neuronal vulnerability in the human patient. For the development of successful therapeutics that promote cognitive health, it is imperative that models with enhanced translational value are utilised. Without such investigations, it is unlikely that rodent models will provide unequivocal delineations of the molecular mechanisms orchestrating primate age-related cognitive decline.
Figure 43: Temporal regulation of proteins commonly identified in mouse, rhesus macaque and human synapses. A. Venn diagram middle intersection displays the 502 commonly identified proteins in the mouse, rhesus macaque and human patient synaptic ageing analyses. B. Heat map exhibits the temporal expression profiles of the 502 proteins during ageing in the mouse, rhesus macaque and human patient. Protein expression values were log2 transformed and analysed by hierarchical clustering. Note the differential expression of proteins in the mouse synaptic ageing timecourse versus the primates. Red represents age-related upregulation of the protein; blue denotes age-related downregulation of the candidate. YS = young synaptic; MS = mid-age synaptic; OS = old synaptic. Input data: mouse = synaptic mitochondrial ageing (Chapter 4); rhesus macaque = hippocampal synaptic ageing (Chapter 5); human = hippocampal synaptic ageing (Chapter 6).

7.3 Further studies

Whilst we have provided several novel comprehensive analyses of synaptic ageing throughout this thesis, there were, of course, time constraints preventing further investigations into the data. As highlighted in Chapter 4, we experienced numerous issues with the mouse samples, including at the proteomic and biochemical level, which resulted in reduced time for quantification and analysis of the data using the systematic approach we had planned. We had initially isolated synaptic and non-synaptic mitochondria from the cortices of 4-week-old mice harbouring the neuroprotective slow wallerian degeneration (Wld⁰) mutation, in order to delineate the inherent molecular mediators that may be conferring synaptic protection. Despite obtaining the proteomic data from the mutant mouse model, we were unable to perform an isolated analysis directly comparing the age-matched wild-type and Wld⁰ mice. These data would have provided an additional dimension to the analysis, enabling examination of the spatiotemporal expression of protective functional mediators in the wild-type mouse and further observing whether alterations in the expression of these candidates modulates increased synaptic vulnerability during advancing age. Furthermore, we had planned to perform in vivo experiments recapitulating the expression of potentially neuroprotective candidates in an attempt to rescue age-induced synaptic phenotypes in Drosophila models. These studies may
offer mechanistic insights into the molecular mediators facilitating synaptic protection and provide novel candidates that may be regarded as therapeutic targets for age-related neurological diseases where synapse loss is particularly problematic. However, as highlighted above, whether these results will be translatable to the human patient remains to be seen.

7.4 Conclusions

Here, we have provided the first comprehensive analyses describing the dynamic proteostatic regulation of discrete mitochondrial and synaptic populations during normal healthy ageing. We demonstrate that the trajectory of ageing appears to display significant malleability suggesting that strategic interventions may prevent age-related cognitive decline and associated neurodegenerative diseases. Further investigations examining the molecular correlates of age-dependent synaptic vulnerability in physiologically relevant models will prove valuable in elucidating the mechanistic cascades attenuating the structure and function of the synapse. Without a comprehensive understanding of the perturbed pathways governing synaptic demise during advancing age, there are limited possibilities for the development of efficacious therapeutic targets promoting cognitive health.
List of Publications


Understanding the molecular consequences of inherited muscular dystrophies: advancements through proteomic experimentation

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1. Introduction

The muscular dystrophies (MDs) are a superfamily of heritable heterogeneous disorders that exhibit similar clinical and pathological features in those affected [1–6]. It is estimated that MDs affect as many as 1 in 6200 people worldwide and costs exceed $1 billion per year in the United States alone [5]. To date, there are upward of 50 discrete diseases, each of which is defined by a distinct genetic mutation and can be inherited as autosomal dominant, autosomal recessive, X-linked or, in rare cases, may develop sporadically [1,5]. Patients commonly present with progressive weakness in the appendicular, axial, and maxillofacial muscles but the age of onset, severity of disease, and concomitant complications vary dramatically between individuals [1,4–7]. The distribution of muscle weakness often promotes distinction between the particular types of disease [1]. As such, MDs have been categorized into various groups based upon clinical and molecular observations; these include but are not limited to Duchenne muscular dystrophy (DMD), myotonic dystrophy type 1 (DM1), facioscapulohumeral muscular dystrophy (FSHD), limb-girdle muscular dystrophy (LGMD), Emery–Dreifuss muscular dystrophy (EMD), and collagen VI myopathies.

Recent advances in molecular genetics have promoted further understanding of the mechanisms governing the varied types of MDs. Studies have identified over 30 causative genes [1–3] that are involved in the pathogenesis of these diseases (DMD: 1 gene; FSHD: 2 genes; LGMD: 25 genes; EMD: 6 genes; collagen VI: 3 genes); most of which appear to be protein coding. Localization studies of MD-related candidates indicate perturbations may occur in the skeletal muscle sarcolemma, nuclear membrane, extracellular matrix, intermediate filament network, and sarcomere [1,3,8]. Despite this, the functions, pathways, and downstream targets of these proteins remain to be elucidated. For efficacious therapeutic targeting of MDs, it is imperative that research focuses on the downstream networks of each particular mutation to assess where intervention may restore cellular homeostasis. Proteomic technologies are well equipped to examine such processes and various laboratories have begun utilizing these techniques for identification of biomarkers and novel remedial candidates in MDs.

In this review, we will outline the relative complexities of studying MDs and how these may be addressed by utilizing modern proteomic approaches. We aim to discuss the current knowledge concerning the most common MD – DMD and some of the less prevalent forms including DM1, FSHD, LGMD, EMD, and collagen VI myopathies. Here, we summarize proteomic-derived advancements in our understanding of these conditions to date and, where possible and/or appropriate, highlight conserved downstream molecular perturbations which may prove...
useful as novel biomarkers for disease progression and future therapeutic investigations.

2. The dystrophies

2.1. DMD

DMD is the most common dystrophy and (to date) the most thoroughly investigated using proteomic methodologies. DMD is a recessive X-linked disease, characterized by muscle degeneration and premature death, typically by the age of 20–30 years. With an incidence of approximately 11–28/100,000 males, DMD is one of the most common and severe types of MD [8]. The cause of DMD is a mutation in the dystrophin gene, leading to an absence of the cytoskeletal protein, dystrophin [9], and subsequent weakening of the structural integrity of muscle cells. The majority of therapeutic approaches for DMD have focused on restoring dystrophin production by modulation of RNA using antisense oligonucleotides [10]. The development of alternative and/or complementary therapeutic strategies to target modifiers of DMD (reviewed by Vo and McNally, 2015 [11]) or the consequence of downstream pathology [12] appears to be emerging areas of research. For work in this area to progress, however, a detailed understanding of the molecules and pathways involved in DMD is required.

2.2. DM1

DM1 typically manifests in early adulthood and is classified as a multisystemic neuromuscular disease [5,13]. It is the second most prevalent dystrophy, but most prevalent adult-onset MD affecting up to 1 in 8000 individuals worldwide [14]. The disease displays an autosomal dominant mode of inheritance and patients often present with highly heterogeneous symptoms including clinical myotonia, progressive muscular weakness, cardiac arrhythmia, visual disturbances, and insulin resistance [5,14]. These diverse phenotypes are caused by a large expansion of the (CTG)n trinucleotide repeat in the 3′ untranslated region of the dystrophia myotonica protein kinase gene on chromosome 19q13.3 [5,13,15]. Patients with substantial amplifications of these CTG repeats demonstrate more severe phenotypes and often present with symptoms at a much earlier age, promoting a diminished life span [14,15]. Broadly speaking, the clinical diversity of DM1 phenotypes appears to stem from the sequestration of mutant RNA transcripts encoded by the CTG expansion [13,15]. These transcripts accumulate within the nuclei of various tissues and promote perturbations in the pathways that regulate alternative splicing programs. Mis-splicing of numerous genes has been experimentally observed in DM1 patient tissues and mutant cell lines suggesting that patient phenotypes may be attributed to the aberrant expression of muscle-specific Cl− channels, cardiac troponin T, insulin receptors, and the sarcoplasmic Ca2+ ATPases [13,15–17]. Although alternative splicing appears to demonstrate some involvement within the development and pathogenesis of DM1, there remains a lack in understanding of how mechanistic pathways could be therapeutically targeted to ameliorate disease progression.

2.3. FSHD

FSHD is the third most prevalent of the MDs and is an autosomal dominant disease with variable penetrance [18,19]. Typically, as the name suggests, patients present with weakness in the maxillofacial muscles and shoulder girdle, which subsequently progresses to affect the pelvis and lower extremities [18–20]. The onset, progression, and severity of FSHD are highly variable between and within families and patients can range from asymptomatic to critical [19]. Unlike other MDs, FSHD usually emerges in adulthood with diagnosis typically occurring in the second or third decade [1,19,20]. However, there are reports of patients presenting with early FSHD symptoms in their 60s and 70s [18–20], highlighting the heterogeneity of the disease.

The clinical variability of FSHD likely stems from the mutation the patient is harboring. The most common form of the disease, FSHD1, is the result of a contraction of microsatellite repeats in the D4Z4 element located on the 4q35 subtelomeric region on chromosome 4 [6,18–21]. Patients typically present with 1–10 D4Z4 repeats whereas the general population demonstrate 11–100 [6,18–21]. Correlations between the repeat size and clinical severity of patients have been reported with those harboring 1–3 copies often more severe than individuals with 8–10 copies [18,19]. Evidence suggests that this reduction in D4Z4 copies induces chromatin remodeling promoting cellular toxicity and degeneration in skeletal muscle [20]. Although there are no obvious mutations in any protein-coding gene, it has been proposed that there may be erroneous activation of the DUX4, FRG1, FRG2, and ANT1 genes that are located centromeric of the D4Z4 array [18,20]. Little is currently known about the molecular cascades that are responsible for the clinical manifestation of FSHD due to the challenging nature of the disease; thus, the identification of therapeutic targets remains in its infancy. Systematic analyses utilizing ‘-omics’ data will be invaluable in the field in order to establish biomarkers of disease and efficacious treatments for FSHD patients.

2.4. LGMDs

LGMDs are a group of inherited diseases characterized by progressive weakness and wasting of shoulder and pelvic girdle muscles. Broadly, there are 2 subcategories of LGMD: those which display a dominant manner of inheritance, termed LGMD1 (upward of 8 subtypes), and those which are recessive in nature, LGMD2 (with approximately 20 subtypes) [22]. The overall frequency of LGMDs is 20–40/100,000 individuals [23] with clinical onset typically occurring during the second decade of life [22]. The most extensively studied subtypes of the disease include LGMD2A, LGMD2B, and LGMD1B and the molecular genetics underpinning these conditions are now beginning to be unraveled. Recent studies have suggested that LGMD2A may be caused by mutations in calcpain-3 [24] which promotes the loss of autocalytic function within skeletal muscle, stimulating fiber degeneration and atrophy [25]. Although there are indications that calcpain-3 is involved in the pathophysiology of LGMD2A, the function of
the protein is still to be established, providing complexities in experimental design and interpretation.

LGMD2B is also believed to be caused by mutations in a calcium-handling protein [26]. Patients presenting with LGMD2B demonstrate mutations in the dysferlin (DYSF) gene [27], which encodes a membrane-associated protein localized to the sarcolemma. Dysferlin has been noted for its capacity to aid in membrane regeneration and impairments in its function appear to stimulate myonecrosis due to increased calcium influx in skeletal muscle. These pathological processes are thought to lead to the characteristic shoulder and pelvic girdle weakness [28] LGMD1B, like subtypes of EDMD, is caused by mutations in the lamin A/C (LMNA) gene [29,30]. Mutations in this gene result in a diverse range of phenotypes often with muscular and/or cardiac involvement; however, it is not clear how LMNA contributes to these clinical manifestations. Studying the molecular pathways involved downstream of LGMD mutations is especially challenging due to the heterogeneity of genetic mutations, complex clinical diagnosis, and availability of human samples.

2.5. EDMD

EDMD affects 1 in 100,000 males [31] and is characterized by scapulohumero-peroneal muscle weakness, joint contractures, and cardiac defects that include arrhythmias and dilated cardiomyopathy [32]. Onset of EDMD is typically seen during childhood or early adolescence [33] and is caused by mutations in various genes that are localized to the nuclear envelope [34]. Commonly, the disease is X-linked recessive and is associated with mutations in the emerin (EMD) gene, which consequently causes the truncation of emerin proteins (in around two-thirds of patients). However, multiple subtypes mediated by a range of genetic mutations in the autosomes also exist (for more information, see Pillers & Bergen [35]). As discussed in Section 2.4, a degree of homology exists between LGMD1B and autosomal dominant EDMD due to both subtypes demonstrating mutations in the LMNA gene. Although it is poorly understood how mutations in lamin A/C contribute to the LGMD phenotype, EDMD is believed to be caused by single amino acid substitutions that result in destabilization of the protein promoting nuclear fragility [36]. Less prevalent autosomal dominant forms of the disease have demonstrated loss of function mutations in the nesprin-1 (SYNE1 gene) and nesprin-2 (SYNE2 gene) proteins (OMIM no. 310300) fostering perturbations in nuclear architecture [37].

2.6. Collagen VI myopathies

Collagen VI is a ubiquitously expressed extracellular matrix protein (ECM) composed of threelfolded chains that form dimers and tetramers. In muscle, the collagen VI network surrounds the basement membrane transferring mechanical and biochemical signals from the ECM to the fiber [38]. Mutations in any of these genes can cause dysfunction in the microfibrilar network in the ECM of muscle, skin, and tendons leading to muscle weakness, joint laxity, contractures, and respiratory compromise [39]. Dominant and recessive mutations in collagen VI are often associated with the COL6A1, COL6A2, and COL6S3 genes and lead to two types of MD: Ullrich congenital muscular dystrophy (UCMD) and Bethlem myopathy (BM) [40–42]. These diseases are relatively rare with an estimated prevalence of 0.1 in 100,000 and 0.5 in 100,000, respectively [43]. UCMD is an autosomal recessive disorder, typically presenting at birth, with infants demonstrating hypotonia and congenital hip dislocation. The majority of patients do not reach the major motor milestones and struggle to walk independently. Accompanying the motor symptoms is severe respiratory problems that require intervention during the first or second decade of life [44]. BM is phenotypically milder than UCMD with patients demonstrating a near normal life span [42,45]. Despite this, it is estimated that 50% of individuals require ambulatory assistance after the age of 50 due to the progressive deterioration of muscle and joint integrity [46]. For a comprehensive review, see Lampe and Bushby [43].

3. Unraveling downstream dystrophic cascades through proteomic investigations

As highlighted in Sections 2.1–2.6, there are numerous MD variants aside from the most well-known DMD, caused by a wide range of associated genetic mutations. There are currently no treatments that ameliorate the neuromuscular phenotype and molecular pathology of any of these diseases [2]. Although several clinical trials for novel therapeutics are in progress, there remains a lack of understanding of the basic molecular biology underpinning these diseases. To identify efficacious pharmacologic targets, it is imperative that the field utilizes modern ‘omic’ technologies to examine the pathways and processes that are perturbed and how these may regulate downstream pathology. This will facilitate a broader understanding of the molecular mechanisms governing muscle development, stability, and pathogenesis and will ultimately enable data-driven interventions that will benefit patients significantly.

As many as 50 discrete diseases fall under the umbrella of the dystrophies but at the time of writing this manuscript, there are only around 26 published proteomic investigations carried out on dystrophy patient or model systems. Of these, 19 studies are focused on the most prevalent and widely known DMD (Supplementary Table 1 [15,18,20,21,39,81–83]) whilst the remaining 8 surround the other less pervasive forms described above (DM1, FSHD, LGMD, EDMD, and collagen VI myopathies; Supplementary Table 3).

3.1. The search for differentially expressed proteins in DMD versus control tissues

To date, approximately 19 separate publications from several different research groups have utilized unbiased quantitative proteomics technologies to identify differentially expressed proteins in models of DMD compared to control subjects. All but one of these studies were conducted in vivo, with diaphragm, cardiac, and various skeletal muscles being the most popular tissue source. Whilst the vast majority of these studies have utilized the mdx mouse model of DMD, material from
Supplementary Table 2: proteins that met these criteria and are summarized in this section will be on proteins that were consistently changed in expression across three or more of the separate proteomic comparisons listed in Supplementary Table 1. This approach also limits the pool of useable studies. For example, although Ge et al. may have carried out the first of these proteomic studies in 2003, we are unable to include their results in our comparative analysis as the data sets are not freely available [50]. Review of these data sets revealed 34 proteins that met these criteria and are summarized in Supplementary Table 2 [49–50,54–56,71–74,92–95,97–100].

3.1.1. Increased expression of structural proteins in DMD
The type III intermediate filament proteins, desmin and vimentin, were consistently increased across 8 and 15 comparisons of DMD tissues, respectively. Considered as a hallmark of developing myotubes [51], the high expression of both proteins has previously been documented in regenerating muscle fibers from different neuromuscular diseases, including DMD [52,53]. Other structural proteins were also consistently increased in DMD tissue across multiple proteomic comparisons, including beta-tubulin, lamin A/C, lamin B1, and spectrin alpha chain, as well as proteins associated with protein assembly (e.g., elongation protein, protein disulfide-isomerase A3) (Supplementary Table 2). One possible explanation for this apparent structural reorganization is that it may represent an attempted compensatory response to stabilize the weakened cytoskeleton [54]. It is interesting to note that increased levels of desmin were also detected in a proteomics study of the mildly affected (and thus, non-regenerating) extraocular muscle (EOM) from the mdx mouse [55], lending support to this notion. The possibility, however, that the changes in structural proteins may merely depict the ongoing process of cellular degeneration and/or fibrosis must also be considered [56].

3.1.2. Cellular stress responses in DMD
Several proteins associated with a cell stress response were elevated in multiple proteomic studies of DMD tissue (Supplementary Table 2), including the heat shock proteins 90, 70, 71, and 78 kDa glucose protein (also known as heat shock 70 kDa protein 5). Thought to represent a molecular response to cell stress, the increased expression of heat shock proteins correlates well with their known involvement in dystrophin-deficient muscles [57].

Increased expression of oxidative stress markers including hemopexin and glutathione-S-transferase (GST) was detected across several different proteomic studies of DMD (Supplementary Table 2). Glutathione metabolism is clearly dysregulated in dystrophic muscle [58,59], but the cause and functional consequences of this are unclear. While one report has proposed a model in which altered glutathione metabolism represents an adaptive and attempted compensatory response to oxidative stress [58], others argue that the dysregulation of this pathway may actually be the cause of increased oxidative stress in DMD [59,60]. Reports of GST activity levels in DMD are also contradictory. While one report demonstrated a marked reduction of GST activity in muscle from DMD patients [59], a study of the chicken model of DMD demonstrated a reduction of activity [61]. Though the biochemical studies above detail the differential expression of several key players in the glutathione metabolism pathway, as well as the activity of GST, the actual protein expression levels of GST have not yet been verified at the biochemical level. Given that elevated levels of GST were detected in four separate proteomic studies of DMD (Supplementary Table 2), it would seem there is an opportunity to examine this further and to determine whether it is possible to alter the capacity of DMD cells to respond to oxidative stress by manipulating GST expression and activity.

3.1.3. Increased membrane permeability in DMD
Increased membrane permeability is a hallmark of DMD and though theories exist about what may cause this (reviewed by Allen and Whitehead [62]), including contraction-induced tears due to fragility of the already weakened membrane, oxidative damage to membranes, or altered regulation of calcium ion channels, the precise mechanisms remain elusive. An increased level of serum albumin was detected across 10 separate proteomic comparisons of DMD versus control tissues (Supplementary Table 2) and likely reflects the increased membrane permeability of the target tissue [56]. Indeed, damage-induced disruption of muscle fiber membranes is commonly associated with an influx of extracellular components, containing albumin, into the muscle [63] and has previously been detected at the histological level in DMD muscles too [64,65].

Parvalbumin, on the other hand, was reduced across eight separate proteomics studies and was one of only two proteins showing a consistent decrease across the multiple proteomic comparisons of DMD and control tissues (Supplementary Table 2). In contrast, a separate proteomics-based biomarker discovery project detected increased parvalbumin levels in mdx mouse sera [66], possibly indicating that the reduction of parvalbumin in DMD tissues may be a result of parvalbumin leaking out into the extracellular space rather than an intracellular-controlled mechanism. Reduced levels of parvalbumin in DMD muscle have also been reported previously from biochemical studies [67,68] and have been implicated in the ‘Ca²⁺ overload theory,’ proposed as a leading mechanism of cellular degeneration in DMD (reviewed by Vallejo-llarramendi et al. [69]). Potential consequences of Ca²⁺ overload were also detected in multiple proteomic comparisons, including an increased expression of the Ca²⁺-binding protein troponin C (three comparisons) and increased expression of the Ca²⁺-effector proteins, annexin 2 (eight comparisons), and annexin 5 (six comparisons) (Supplementary Table 2). Does the influx of albumin, another Ca²⁺-binding protein, also contribute to Ca²⁺ overload mechanisms in DMD tissues?

3.1.4. Immune cells may contribute to the proteome of DMD tissues
A prominent feature of DMD muscle is the presence of an obvious immune response, though the functional consequences of this are still a matter of debate [70]. Several
types of immune cells have been shown to infiltrate mouse and human DMD muscle, including macrophages, eosinophils, natural killer T cells, CD4+, and CD8+ T cells [70]. Whilst proteomics comparisons may have revealed insights into the molecular response to this influx (e.g. increased levels of leukocyte elastase inhibitor A (Supplementary Table 2)), it is important to consider that each of these immune cell types could potentially contribute a unique repertoire of proteins – quite different from the muscle itself – and would thus skew the proteomic profile of the sample. Western blot verification of differential protein expression from total protein extracts appears to have been common practice but few studies of this nature have also provided histological analysis of the same proteins, meaning that any changes in protein expression that are related to tissue heterogeneity would likely be unattributed.

3.1.5. Proteomic insights into the differential vulnerability of muscles in DMD

Of note is that there are a large number of proteins (50+) detected across the studies of the proteomic investigations (summarized in Supplementary Table 1) that showed contradictory patterns of expression in different comparisons (i.e. increased in expression in one or more proteomic comparisons but decreased in others). Examples of such proteins are GAPDH, various myosin chains, creatine kinase, glycogen phosphorylase, myoglobin, and adenylate kinase (also identified in [71]). As alluded to previously, changes in the levels of some of these proteins could be ‘false positives,’ arising from variations in disease models, tissue heterogeneity, or tissue sampling techniques. There is also the possibility, however, that some of these differences may be useful for determining which constitutive and/or adaptive molecular pathways contribute to the differential vulnerability of different muscles in DMD.

Two of the proteomics studies listed in Supplementary Table 1 specifically aimed to shed light on the molecular pathways that determine how vulnerable a particular muscle type is to an absence of dystrophin. A 2-dimensional difference in gel electrophoresis-based quantitative proteomics comparison of the mildly affected EOM from mdx and control mice revealed differential expression of just seven proteins [55]. The authors highlight how these results are a stark contrast to previous 2D-gel-based comparisons of the severely affected diaphragm muscle, where between 20 and 35 differentially expressed proteins were detected [72,73]. This suggests that there is a minimal perturbation of molecular pathways in the EOM muscle and perhaps also implies that adaptive molecular pathways may not extend far beyond a straightforward upregulation of the dystrophin homolog, utrophin [55].

A later study from the same group – in which the proteome of the soleus (SOL), extensor digitorum longus (EDL), flexor digitorum brevis (FDB), and interosseous (INT) muscles from the mdx mouse was quantitatively compared with control mice – also found differences between the number of differentially expressed proteins across different muscle types [74] (see Supplementary Table 1). The histological analysis revealed a higher degree of hypertrophy and central nucleation (a hallmark of muscle fiber regeneration) in the SOL and EDL muscles compared to INT and FDB. This clearly does not consistently correlate with the degree of differential protein expression, however, since just 5 proteins were differentially expressed in the INT but 19 were differentially expressed in FDB (i.e. more than the number detected in the EDL muscle; Supplementary Table 1). Notwithstanding the potential limitations of 2D-gel-based quantitative proteomic comparisons, these findings serve to highlight the importance of considering results from multiple tissue types before drawing generalizable mechanistic conclusions about DMD.

3.1.6. Temporal proteomic studies of DMD

Several proteomic studies aimed to identify temporal changes during disease progression in DMD mouse models. These studies provide insights into the longer term secondary molecular changes that occur during disease progression in mdx mouse hindlimb muscle [71], cardiac muscle [75], tibialis anterior [76], and diaphragm [77].

Some potentially interesting differences were detected in aged hearts from mdx mice, including a reduction of lamin A/C, vimentin, and annexin [75], that were both undetected in the other aging studies and contrast with findings from various DMD versus control comparisons (Supplementary Table 2). Though the authors were unable to verify the reduction of lamin A/C and vimentin by Western blot, reduced levels of annexin were confirmed. In addition, while the expression level of the developmentally regulated protein, myosin light chain 2, was consistently increased in mdx mouse hindlimb muscle at 1, 3, and 6 months of age compared to age-matched controls [71], the levels in aged mdx mouse hearts appear reduced compared to controls [75]. It is clearly not possible to draw direct comparisons between the various studies because of differences in the age of the tissue being compared, but it would be interesting in the future to determine whether the differential expression of such candidates offers insights into mechanisms underlying the differential vulnerability of muscles in DMD.

3.2. Proteomic insights into other MDs

In recent years, several studies utilizing proteomics have appeared in the literature surrounding these less prevalent MDs with the aim of enhancing our understanding of the molecular mechanisms underlying the downstream effects of the causative mutations. The majority of these investigations have employed human patient muscle tissue for comparative characterization of protein expression versus controls, in an attempt to distinguish groups of dysregulated proteins in various MD subtypes. This has allowed the generation of lists of differentially expressed proteins, providing indications of the biological functions and pathways involved in the pathogenesis of various MDs. However, there is a requirement for larger numbers of well-executed studies to dissect cause/consequence relationships and determine which alterations may reflect conserved responses in the range of diseases.

The complexity of the genetic background of each disease as well as the limited availability of human donors provides challenges. Currently, there are only seven proteomic-based studies focusing on the diseases discussed in Section 2 above,
most of which have utilized 2D gels, followed by mass spectrometry (MS) analysis. Selected reports discussed here have attempted to discern the commonalities and differences in protein expression between genetic variants of particular MDs as well as between diseases using these techniques (see Supplementary Tables 3 and 4 for a summary).

3.2.1. DM1

There is only one readily available publication employing proteomic techniques in an attempt to identify the molecular cascades which are perturbed downstream of the causative genetic insult in DM1. Hernández-Hernández and colleagues [15] utilized a 2D-gel-based proteomic analysis on DM1 transgenic mice with 45 kb of human genomic DNA originally cloned from a patient with DM1 [78,79]. Here, they identify potential alterations in post-synapsin I (SYN1) translational modifications and elements of RAB3a and its downstream cascades. Various RAB alterations have been associated with other neurodegenerative conditions including retinopathies [80], suggesting the possibility of conserved mechanistic cascades across multiple apparently unrelated neurodegenerative conditions. However, this study is limited by its choice of controls, depth of coverage granted by the use of 2D gels and lacks clarity in terms of identification of sample type used for the experiments.

3.2.2. FSHD

At present, there are three proteomic studies attempting to address distinct molecular alterations that may be specifically associated with FSHD. An early study performed by Celegato et al. [18] utilized 2 dimensional electrophoresis (2-DE), high performance liquid chromatography–MS, and transcriptomic methods to characterize deltoideus muscle protein expression in groups of patients (aged 8–69 years) with varying D4Z4 repeat lengths. The group identified a common profile of proteins associated with FSHD, independent of repeat size, suggesting proteins associated with glycolysis, the tricarboxylic acid cycle, and protein synthesis (particularly, elongation factor Tu) are upregulated in patient versus control samples; conversely, detoxification and degradation proteins (SOD, PRDX2) and actin isoforms are downregulated in FSHD patients. Proteins involved in muscle differentiation also appeared to demonstrate differential expression between FSHD patient groups and controls: these included COP9, HSP27, alpha-crystallin B, phosphoglycerate mutase, creatine kinase, and myosin heavy-chain proteins. Upon further analysis, the study identified a conserved upstream regulator – MyoD, levels of which were shown to be consistently reduced in patients. The authors hypothesized that defects in MyoD signaling promoted the failure of regeneration of fast glycolytic muscle fibers after episodes of mechanical stress, leading to a progressive increase in slow oxidative fibers, promoting weakness and dystrophy in FSHD patient muscle.

In a similar study by Laoudj-Chenivesse et al. [21], 2-DE proteomics coupled with MS also identified alterations in the detoxification and oxidative stress machinery in FSHD patient muscle biopsies. The specimens were obtained from the deltoideus and quadriceps muscles and included a range of individuals (aged 17–66 years), all demonstrating various D4Z4 repeat lengths. Although there appear to be overlaps in the pathways detected between the Laoudj-Chenivesse et al. [21] study and the work performed by Celegato et al. [18], the directionality of the protein expression alterations contrasts. For instance, Celegato et al. [18] report the down-regulation of proteins associated with detoxification processes, whereas Laoudj-Chenivesse et al. [21] demonstrate a significant upregulation of these cascades (proteins include SOD1 and GST). Due to the reported upregulation of oxidative stress markers, the Laoudj-Chenivesse et al. [21] study focused on the potential impact of mitochondrial dysfunction on muscle fiber integrity that was hypothesized to be regulated by increased ANT1 expression – a gene neighboring the D4Z4 repeat locus.

The final study concerning FSHD has provided another perspective on the molecular pathogenesis of the disease. Tassin et al. [20] utilized patient-derived myoblasts (n = 2) and gel-free shotgun proteomics (2DLC–MS/MS) to characterize atrophic and disorganized FSHD myotubes versus control cells. In total, 336 proteins were quantified from the quadriceps-derived myoblasts with the study illustrating that myosin heavy and light chain (MYH8, MYH3, MYH7; MYL1, MYL6B) and caveolar proteins appeared dysregulated in primary FSHD cells. The authors highlighted caveolin-3 (CAV3) and its associated networks as potentially perturbed in FSHD promoting the reduction of myogenic differentiation in skeletal muscle. CAV3 mutations have previously been documented in other neuromuscular diseases, including LGMD1 and LGMD2B, which may suggest that caveolin dysregulation is a consequence of myotube degeneration as opposed to an upstream regulator of FSHD.

3.2.3. Molecular overlaps across multiple dystrophies

There is a requirement for further comparative studies in order to elucidate how these membrane micro-domains may play a role in pathogenesis. One such study by De La Torre et al. [81] similarly documented an impairment in myotube differentiation in LGMD2B patient muscle biopsies. This comparative investigation focused on differentially expressed proteins between LGMD2A, LGMD2B, FSHD, and control triceps and quadriceps muscle using 2-DE and MALDI–TOF MS. The authors provided details on 17 conserved proteins that appear altered in all the neuromuscular diseases characterized versus the control samples. These proteins displayed involvement in energy metabolism, the myofibril, and muscle development and repair, agreeing with the previously discussed manuscripts studying FSHD. Much like the Celegato et al. investigation [18], the group shows alterations in the muscle fiber distribution with a significant increase in slow-twitch fibers. These remodeling events appear to be occurring in numerous neuromuscular diseases and track with disease progression. The authors also elaborated to include proteins that demonstrated alterations specifically in LGMD2B patients. These 14 candidates exhibited similar functional categories to those 17 that were conserved through the neuromuscular diseases examined. Although in the De La Torre et al. study [81] these proteins appeared to demonstrate unique alterations in LGMD2B patients, upon further inspection of the literature, there are indications that several of these candidates have been discussed in a range of neuromuscular diseases including collagen VI.
myopathies (De Palma et al. [39]), FSHD (Celegato et al. [18]), and Duchenne MD (see Supplementary Table 4).

Considerable overlaps exist between investigations examining FSHD and LGMD subtypes, which may be due to the upstream regulators of disease or the conserved downstream processes of muscle degeneration. Magagnotti et al. [82] also noted similar pathways may be disrupted in EDMD with patients harboring mutations in the LMNA gene, namely LGMD1B. 2-DE proteomics, MALDI–TOF MS, and in silico analyses of patient fibroblasts suggested that proteins regulating cytoskeletal/structural organization were less abundant in individuals with a diagnosed laminopathy. Conversely, and in keeping with the Laoudj-Chenivesse et al. FSHD study [21], oxidative stress markers appeared enriched in patient cells versus controls. Despite Magagnotti et al. [82] utilizing groups of patients with general myopathies as an internal control to assess specific protein alterations in laminopathies, it is clear from examination of multiple published data sets that several of these candidates have been reported to be differentially expressed in other neuromuscular diseases (see Supplementary Table 4 [18,20–21,39,49,54–56,72–74,81–83,92–94,99]).

Suggestions of skeletal muscle remodeling in MDs are frequently referenced in the literature due to the dynamic alterations in proteins involved in myofibrillar architecture and cytoskeletal integrity (Celegato et al. [18], Tassin et al. [20], De La Torre et al. [81], Magagnotti et al. [82] – see Supplementary Tables 2 and 4). De Palma et al. [83] also proposed that in LGMD2B patients, there was a redistribution of muscle fiber type as proteins involved in oxidative phosphorylation were increased and those associated with anaerobic metabolism decreased versus control samples. In accordance with the alterations in expression of bioenergetic candidates, myosin light-chain isoforms were also differentially expressed in the LGMD2B patients’ quadriceps muscle, suggesting functional impairments in contractile velocity and force may be due to increased numbers of slow-twitch fibers.

A further study conducted by the same authors (De Palma et al. [39]) focused on the collagen VI myopathies: UCMD and BM (see Section 2.6). Human quadriceps muscle biopsies from BM (n = 8), UCMD (n = 4), and control (n = 2) patients revealed bioenergetics pathways were altered in both BM and UCMD individuals versus healthy samples. The downregulation of the hexosamine biosynthetic pathway (HBP) was highlighted as a key driver of BM and UCMD progression due to associations with protein homeostasis in the endoplasmic reticulum and unfolded protein response systems. Although this reduction in the HBP and glycosylation appeared conserved between the collagen VI MDs, the downstream biochemical alterations in UCMD and BM displayed unique properties, likely reflecting the differences in disease severity. In BM patient samples, the authors suggest that the muscle protein quality control system is sustained by metabolic adaptation. This allows the cells’ energy requirements to be met and the catastrophic consequences of the ER protein misfolding response to diminish. In contrast, UCMD patients demonstrate disruption in this pathway and the compensatory-layered mechanism, likely leading to lipotoxicity and cellular apoptosis. Interestingly, other MDs such as spinal muscular atrophy (SMA) demonstrate perturbations in proteostasis with ubiquitin homeostasis defects influencing neuromuscular pathology [84] (see Section 2.1).

3.2.4. In silico analysis suggests proteomic studies are highlighting downstream consequences of degenerative cascades

Although the appearance of common themes in the MD field may permit enhanced understanding of the molecular pathology of the various diseases, it may also be a great hindrance. The proteomic studies discussed examined groups of heterogeneous conditions caused by numerous discrete genetic mutations that all encode for different proteins, pathways, and processes (see Section 2). Thus, the emergence of these conserved changes in cytoskeletal and bioenergetic families throughout various neuromuscular disorders suggests that these alterations are likely a downstream consequence of causal upstream perturbations. Many alterations are likely an adaptive response to ongoing myofibril degeneration – a process occurring in all MDs discussed. In fact, with in silico analysis of the proteins identified in Supplementary Table 4, there are clear indications that a substantial number of these candidates may be involved in downstream degenerative cascades occurring in a wide range of tissues – not merely myofibrils (Figure 2(a)). Alterations in expression of upward of 15 of these proteins have been associated with Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and motor neuron disease in published manuscripts (Figure 1(b)), illustrating likely late-stage consequences of cellular dysregulation. Thus, it is probable that these candidates do not represent viable therapeutic targets or biomarkers for MDs. Despite this, vimentin is consistently identified as upregulated throughout the different MD studies. Interestingly, vimentin also appears to lie upstream of the majority of the candidates identified by the investigations reviewed here (Figure 1(c)). These observations may warrant further analyses into what lies further upstream of vimentin and how this may be potentially promoting dysregulation selectively within the myofibrillar architecture. However, in order to successfully elucidate the upstream regulators of various MDs, there are several considerations that require attention before experimentation. Fortunately, the field is now in the position to successfully draw on lessons learned from other fields (such as SMA), where considered applications of proteomic techniques have yielded tangible gains (Figure 2).

4. Future directions in dystrophy research through proteomic investigations

The data sets reviewed in Section 3 provide novel and valuable insights into the molecular pathways that may be disrupted in MDs. The search for conserved and unique molecular alterations in MD variants has begun to shed light on the downstream pathways affected by these discrete mutations. Despite this, the current studies demonstrate some shortcomings in proteomic experimental design and data analysis that should be addressed.

4.1. Tissue selection and characterization

The investigations discussed in this review appear to focus on static stages of disease in a variety of animal model or pooled patient samples. This is a fundamental flaw whilst attempting to
elucidate biomarkers of disease progression because this approach does not account for the numerous variables that may have influenced the data acquired. Primarily, there appears to be an oversimplification of proteomic investigations, leading to the loss of potentially relevant information that may indicate how MDs are regulated. Commonly, the studies report two-way comparisons of pooled samples: disease versus control patients; however, the patient cohorts vary significantly. Within the MD pooled patient (and animal model) samples, authors include tissues from a variety of muscles, ages, clinical severities, and genetic mutations – promoting substantial heterogeneity and preventing the possibility of also running a variable-matched control sample. It is now well established that different tissues and even various anatomical regions of the same tissue sample (e.g. proximal versus distal) do not display uniformity in protein expression [85]. Thus, pooling or comparing numerous biopsies from a wide selection of skeletal muscles and cellular populations will only hamper the identification of proteins regulating pathogenesis.

In contrast to the majority of studies conducted on other MDs, the vast majority of proteomic investigations into DMD have utilized tissue from the mdx mouse and whilst two proteomics studies of DMD patients have been conducted, one utilized serum [47] and the other analyzed urine [48]. These sources can certainly be useful for identifying easily accessible biomarkers of disease as demonstrated by Coenen-Stass and colleagues’ [86] innovative identification of peripherally accessible biomarkers which demonstrate response to therapeutic attempts in mdx mice. However, such peripherally accessible samples do not necessarily offer easily translatable mechanistic insights into disease pathology. For example, while one protein may appear elevated in the serum of DMD patients, its expression in the primary tissue (i.e. muscle) could be entirely the opposite. In order to determine that alterations in protein expression are due to the presence of disease, the same ages, sexes, and clinical severities of patients, as well as muscle and, ideally, the same portion of muscle, must be utilized in the pooled MD and control samples. Furthermore, in order to understand disease mechanisms, it is imperative that protein expression in individuals without the condition is characterized so analogous alterations can be eliminated as pathogenic.

4.1.1. Characterization of protein expression profiles throughout disease progression

The degenerative process displays complex and dynamic spatiotemporal molecular profiles, which demonstrate variability

Figure 1. Pathways analysis of conserved overlaps in Muscular Dystrophies. (a) Top canonical pathways bar chart highlighting the main disrupted cascades in multiple MD subtypes (data from supplementary table 4). By combining data from several proteomic studies we are able to identify pathways such as multiple Rho-related cascades or ‘Clathrin-mediated endocytosis signalling’ which were not specifically reported to be disrupted in these studies. (b) Network highlighting how candidates identified in multiple MD proteomic studies interact with other neuronal/neuromuscular diseases. Alzheimer’s, Parkinson’s, Huntington’s and X-linked hereditary diseases demonstrate similar protein expression alterations suggesting these proteins may be involved in downstream degenerative cascades. (c) Top identified network in IPA generated from MD candidates. Vimentin (VIM) appears as a central hub of the network impacting to multiple downstream proteins. Lines in blue indicate VIM interactions with other proteins with a conserved change in multiple datasets. (b–c) All proteins listed in supplementary table 4 were included in the IPA analysis. Candidates with reported quantitative values in >2 studies and demonstrating consistent alterations (up-/or down-regulation) across >50% of these studies were considered for the IPA statistical testing. These proteins were assigned an arbitrary fold-change value of +2 or −2 respectively, for the generation of IPA data. Red = up-regulation; green = down-regulation; grey = proteins not considered for statistical analysis. Dotted lines indicate direct interactions; dashed lines, indirect interactions. (Full color available online).
throughout disease progression dependent upon the upstream genetic mutation. Fluctuations in protein expression throughout the disease course indicate tissue-specific cascades, with differing biochemical alterations often occurring in neighboring populations of cells [87]. These varying protein expression profiles often reflect the vulnerability status of particular cellular clusters that display an enhanced response to insult. In order to determine how alterations in protein expression may modulate cellular and tissue vulnerability, it is important to track candidate alterations through the time course of MDs – from early presymptomatic time points to end-stage disease. There is abundant evidence to suggest that alterations in causative upstream molecular cascades begin long before the onset of detectable pathology [84]. From our own studies, we have observed significant up/downregulation of numerous proteins during the early stages of disease but at later time points, protein expression is quite the contrary [87]. Therefore, focusing on the early stages of disease may provide an enhanced understanding of the molecular mechanisms governing muscle degeneration and offer a viable data source for the identification of novel drug targets. Additionally, a comparison of multiple disease variants (i.e. Duchenne vs. Becker–Kiener dystrophinopathy) with differing severity may also offer more tenable insights into potential upstream moderating and/or regulating molecular cascades.

4.1.2. Proteomic techniques

The availability of modern proteomic techniques is beginning to direct the field away from 2D gels. Tools such as label-free proteomics as well as labeled approaches including isobaric tags for relative and/or absolute quantitation (iTRAQ) or tandem mass tagging enable a more comprehensive characterization of the molecular alterations occurring throughout disease progression. Label-free techniques enable comparative analyses of multiple samples with low concentrations of protein extracts [87,88], which may be beneficial when working with precious resources such as human patient samples. These techniques enable analysis with as little as 3–5 µg of material for injection into an orbitrap. There are of course limitations with the existing tools such as limited dynamic range, compression of ratios calculated for tagged samples, and even something as basic as coverage of the proteome when compared to more established transcriptomics. Whilst transcriptomics may be ahead of proteomics in coverage and usability, it is protein and not RNA which are the ultimate effector molecules and the two do not necessarily correlate well [89]. Therefore, continued developments for the field of proteomics in software (such as Progenesis) that allow the processing and analysis of complex timecourse profiles and/or comparisons, facilitate improved methodologies in the MD field.

4.1.3. Data analysis

Filtering and refining of proteomic data is absolutely essential. There remains a requirement for laboratories to follow standardized criteria in order to provide more reliable and comparable analyses in publications. For example, posttranslational modifications (PTM) and distinct isoforms should be reported (if known), as they may be a source of contradictions
in the data shown. If the technique/software/database for the identification of protein used is not able to distinguish between protein isoform/PTM, strict filtering should be applied to avoid low-quality identifications. The re-reporting of published data sets without reanalysis has the potential to propagate erroneous conclusions throughout published literature within the field. Investigators should also utilize available software for in silico analyses. These tools enable unbiased comprehension of the pathways and processes that may be altered within the samples analyzed. It has become increasingly clear that discrepancies exist in the data reported by investigators and this affects the outputs from independent pathway analyses (see Supplementary Tables 2 and 4 and Figure 1).

5. Five-year perspective on advancements in MD research

The relatively recent advances in proteomic tools and techniques (as discussed in Section 4), coupled with the broad range of disparate mutations leading to multiple forms of dystrophy with varying prevalence, have (to date) severely impeded a coherent approach to the molecular characterization of the downstream molecular cascades regulating the vulnerability of distinct muscle populations and the progression of individual disease variants. We have outlined various experimental obstacles in Section 4, which require attention before conducting proteomic experiments (Figure 2).

There is clearly scope for future work in this area, using modern approaches such as iTRAQ or label-free MS, to quantitatively compare the proteome of MD muscles that show differential vulnerability. Indeed, recent publications examining other neurodegenerative conditions such as the childhood motor neuron disease SMA have taken a concerted proteomic molecular genetic approach to identify regulators pathways and therapeutic targets [84]. Here, the authors applied iTRAQ proteomics to vulnerable neuronal populations in a SMA murine model to identify differentially expressed proteins. Selected candidates were examined for their ability to regulate neuronal stability in multiple small animal model systems including Drosophila and zebrafish before scaling back up to murine systems providing a complete rescue of the neuromuscular system. As a result, the authors have published the most effective non-survival motor neuron replacement therapy to date. The samples and models exist within the dystrophy field to allow the replication of such proven target-rich workflows to be implemented to inform novel (non-replacement-based) therapeutic interventions for the dystrophies.

6. Expert commentary

Current proteomic studies regarding inherited MDs are unraveling common/specific disrupted pathways in terms of the molecular constituents altered, but these candidates are not necessarily altered in a consistent manner due to the nature of the investigations/experimental design employed. Though they provide a window for a better understanding of the process of degeneration, some issues should be addressed in future work. The development of novel proteomics, such as label-free techniques, facilitates a more complex experimental design where factors such as tissue-specific vulnerability and disease stages may be taken into account. This will allow researchers to distinguish between early and late responses of the specific mutations causing MDs and a more accurate mapping of the dynamic processes taking place in the muscle. Moreover, the production of animal and cellular models that faithfully recapitulate the disease phenotype seen in patients will also help for a more comprehensive characterization of the molecular changes taking place throughout disease progression than can later be correlated to human disease. As there are currently no effective therapeutics for the dystrophies, the field is reminiscent of where the SMA field was 10 years ago, i.e. gene replacement therapy is on the extreme horizon, but the tools and techniques are available to make some tangible headway into our understanding of the disease processes underpinning the condition leading to the identification of novel potential non-gene replacement therapeutics along the way [84,87].

Key issues

- Proteomics is a powerful tool for the identification of biomarkers and therapeutic targets.
- Investigators must endeavour to utilise strict and standardized methodologies for comparison of control and disease tissues.
- If identified candidates are enzymes or have a role in metabolic processes, ex vivo biochemical or in vivo reporter assays (in model organisms i.e. Drosphila) should be performed to determine if detection of altered abundance correlates with altered activity/function.
- Studies should utilise western blotting and immunohistochemical analysis, as well as multiple model organisms for validation of candidate relevance to human physiological alterations and to assess their ability to moderate disease processes in vivo.
- Such candidates should be assessed for their ability to moderate disease processes in vivo and in multiple organisms (i.e. Drosphila/Zebrafish/Rodents) in order to confirm relevance in a species/model independent manner.

Acknowledgements

The authors wish to apologise to anyone whose published work we were unable to discuss due to space constraints or was omitted inadvertently. The authors would also like to thank Prof T H Gillingwater (Anatomy, University of Edinburgh) for advice and intellectual support.

Declaration of interest

H Fuller is grateful to the SMA Trust (UK) for providing grant funding for her work on SMA. LC Graham is supported by a BBSRC DTP PhD studentship. M Llavero Hurtado is supported by a Darwin Trust PhD studentship. TM Wishart is funded by the BBSRC (Roslin Institute strategic programme grant - BB/ J004332/1) and the MRC (MR/M010341/1). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.
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Appendix 2

Graphs display the temporal expression profiles of the 451 candidates identified by the Biolayout Express\textsuperscript{3D} analyses. Schematics were constructed using Python Jupyter Notebook. All graphs display the ratio of candidate protein expression against the young age. Synaptic mitochondrial temporal protein expression - blue; non-synaptic temporal protein expression – green; red lines indicate 20% change.
Appendix 3

Graphics demonstrate the presence of polymer smears (indicated by arrows) in the young synaptic mitochondrial sample (top) and difficulty with protein identification due to low alignment scores between samples (bottom).
Appendix 4

Graphs display the temporal expression profiles of the 583 candidates identified by the Biolayout Express\textsuperscript{3D} analyses. Schematics were constructed using Python Jupyter Notebook. All graphs display the ratio of candidate protein expression against the young age. Hippocampal temporal protein expression - blue; occipital cortex temporal protein expression – green; red lines indicate 20% change.
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