Mitochondrial Abnormalities in PrP-null mice and
MeCP2-null mice

Andrew William James Paterson

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Declaration:

I declare that:

- This thesis was written by me
- That the data contained therein comprises of my own work
- That this work has not previously been submitted for any other degree or professional qualification.

Andrew Paterson
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Abstract:
The transmissible spongiform encephalopathies (TSE's), also known as prion diseases, are believed to arise following the conversion of the endogenous cellular prion protein, PrP\(^C\), to a pathogenic isoform, PrP\(^Sc\). Although the physiological function(s) of PrP\(^C\) remain uncertain, recent reports have demonstrated increased levels of oxidative stress markers and mitochondrial abnormalities in PrP-null mice. This thesis will demonstrate further evidence of significant mitochondrial abnormality in PrP-null mice. In addition, the opportunity arose to study mitochondrial function in MeCP2-null mice, a model of the neurodevelopmental disorder Rett syndrome. A mitochondrial abnormality has long been postulated as a contributing factor in Rett Syndrome and altered transcription levels of mitochondrial respiratory Complex subunits have recently been identified in the MeCP2-null model. This thesis will confirm and describe significant mitochondrial pathology in this model.

The phenotype associated with the PrP-null mouse has proved to be subtle, the animals exhibiting minor behavioral modification and controversial electrophysiological differences. There is also a growing body of evidence implicating the involvement of oxidative stress in both TSE's and the PrP-null model. Recent studies of PrP-null mice have uncovered alterations in mitochondrial morphology, MnSOD levels and some weak evidence of altered transcription levels of some subunits of respiratory chain Complexes. Additionally, electrophysiological studies of hippocampal slices, which synaptically express PrP\(^C\) at high levels, have recently shown a reduction in post-tetanic potentiation, which is also suggestive of altered mitochondrial function in the brain. Experiments were therefore conducted to augment this body of evidence in PrP-null mice. The sensitivity of hippocampal slices to experimental tissue culture and the oxidative nature of the culture environment together with basic measures of synaptic efficacy were carried out to investigate the possibility of an oxidative stress-related phenotype for the PrP-null mouse. Evidence of a mitochondrial abnormality in PrP-null mice was sought by extensive assessment of the morphological and functional characteristics of isolated brain mitochondria.
Study of isolated mitochondrial suspensions by electron microscopy revealed that PrP-null mitochondria were larger and, when cristae density was quantitatively measured using a novel technique, to have a reduced cristae density when compared to controls.

Measurement of oxygen consumption by the mitochondrial electron transport chain of PrP-null mice was carried out using mitochondrial and submitochondrial particle suspensions in a Clark-type Oxygen Electrode. A significant increase in respiratory capacity was detected in PrP-null mitochondria when metabolising Complex I substrates, but not when electrons entered downstream of Complex I. This implicates Complex I as a site for pathological change. Analysis of mitochondrial coupling indicated no difference between the genotypes, suggesting that the permeability of the inner mitochondrial membrane to protons was unchanged.

Assessment of superoxide production by the respiratory chain using tempone-H spin-trapping and electron paramagnetic resonance (EPR) spectroscopy proved impossible using intact mitochondria due to interference by endogenous antioxidant systems. However the data suggested that tempone spin-trapping and EPR may yield novel methods for the assay of mitochondrial antioxidants. The mitochondrial antioxidant effect was circumvented by the use of submitochondrial particles (SMP) which lacked the interfering antioxidant system. EPR experiments demonstrated maximal superoxide production by Complex I was significantly increased by around 40% in PrP-null SMP suspensions whilst Complex III superoxide production was unchanged.

As recent studies of the Mecp2-null mouse model of Rett syndrome detected increased transcription of Uqcrcl (which encodes a core subunit of Complex III), mitochondrial morphology and respiration were studied in Mecp2-null mice. Whilst electron microscopy did not reveal any gross alterations in mitochondrial size or cristae density, respiration measurements revealed a severe phenotype in symptomatic, but not presymptomatic, Mecp2-null mice. Mitochondrial coupling was considerably reduced in
isolated brain mitochondria from Mecp2-null mice indicating reduced mitochondrial efficiency. This effect was accompanied by an increase in respiratory capacity through Complex III. To determine if the overexpression of *Uqcrcl* was causative in the production of the observed increase in respiratory capacity, the respiration rates of N2A cells overexpressing *Uqcrcl* were measured. As transfected N2A cells showed significantly increased respiration rates through Complex III, the up-regulation of *Uqcrcl* may be causative in producing the respiratory capacity increase observed in the animal model.

These results enhance the evidence for dysfunctional mitochondria in both PrP-null and Mecp2-null mouse models and are discussed in relation to other investigations of prion disease, Rett syndrome and other models of neurodegenerative disease.
Abbreviation List:

- **aCSF** – Artificial Cerebrospinal Fluid
- **ADP** – Adenosine Diphosphate
- **ALS** – Amyotrophic Lateral Sclerosis
- **ANOVA** – Analysis Of Variance
- **ATP** – Adenosine Triphosphate
- **AUC** – Area Under the Curve
- **BSA** – Bovine Serum Albumin
- **BSE** – Bovine Spongiform Encephalopathy
- **CCS** – Copper Chaperone for SOD1
- **CCD** – Charge Coupled Device
- **CDNB** – 1-Chloro-2,4-Dinitrobenzene
- **CJD** – Creutzfeldt-Jakob Disease
- **CMH** – 1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine
- **CNS** – Central Nervous System
- **3-CP** – 3-Carboxy Proxyl
- **CP-H** – 1-Hydroxy-3-Carboxy-2,2,5,5-Tetramethylpyrrolidine
- **DDRT PCR** – Differential Display Polymerase Chain Reaction
- **DEPMPO** – 5-(Diethoxyphosphoryl)-5-Methyl-1-Pyrroline-N-Oxide
- **DMEM** – Dulbecco’s Modified Eagle Medium
- **DMPO** – 5,5-Dimethyl-1-Pyrroline-N-Oxide
- **DOC** – Deoxycholate
DPI – Dots Per Inch
EDTA – Ethylenediaminetetraacetic acid
EGTA – Ethylene Glycol-bis(Beta-Aminoethyl ether)-N,N,N’,N’-Tetraacetic Acid
EM – Electron Microscopy
EPR – Electron Paramagnetic Resonance (Spectroscopy)
EPSP – Excitatory Post Synaptic Potential
ETC – Electron Transport Chain
FCCP – Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
fEPSP – Field Excitatory Post Synaptic Potential
FFI – Familial Fatal Insomnia
FSE – Feline Spongiform Encephalopathy
FSI – Fatal Sporadic Insomnia
GFAP – Glial Fibrillary Acidic Protein
GPI – Glycosylphosphatidylinisotol
GSH – Reduced Glutathione
GSS – Gerstmann-Sträussler-Scheinker Disease
HEPES – 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
I_{AHP} – After-hyperpolarisation current
L-NAME – Nitro-L-Arginine Methyl Ester
LRP/LR – 37kDa/67kDa Laminin Receptor
LTP – Long Term Potentiation
MDA – Malondialdehyde
MECP2 – Methyl CpG binding protein 2 (human gene)
MECP2 – Methyl CpG binding protein 2 (human protein)
MeCP2 – Methyl CpG binding protein 2 (mouse protein)
MMP – Mitochondrial Membrane Potential
MOPS – 3-(N-Morpholino)propanesulfonic acid
MWCO – Molecular Weight Cut-Off
N2A cell – Neuro-2A cell
NADH – Nicotinamide Adenine Dinucleotide reduced form
NBT – Nitro-Blue Tetrazolium
NCAM – Neural Cell Adhesion Molecule
NOS – Nitric Oxide Synthase
iNOS – Inducible Nitric Oxide Synthase
nNOS – Neuronal Nitric Oxide Synthase
PBKCl – Phosphate Buffered Potassium salt
PCR – Polymerase Chain Reaction
PI – Propidium Iodide
PINK1 – PTEN induced putative kinase 1
PPF – Paired Pulse Facilitation
pr – Probability of Release
PrP – Prion Protein
PrP<sup>C</sup> – Prion Protein normal isoform
PrP<sup>Sc</sup> – Prion Protein scrapie isoform
PTP – Post-Tetanic Potentiation
RCR – Respiratory Control Ratio
RNS – Reactive Nitrogen Species
ROS – Reactive Oxygen Species
SEM – Standard Error of the Mean
SIN-1 – 3-Morpholinosydnonimine
SMP – Submitochondrial Particle
SOD – Superoxide Dismutase
STI1 – Stress inducible protein 1
TCA – Trichloroacetic acid
Tempone – 4-Oxo-2,2,6,6-tetramethylpiperidine-1-oxyl
Tempone-H – 1-Hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine
TME – Transmissible Mink Encephalopathy
TMIO – 2,2,4-Trimethyl-2H-imidazole-1-oxide
TMPD – N,N,N’,N’-tetramethylphenylenediamine
TSE – Transmissible Spongiform Encephalopathy
Uqcrcl – Gene encoding Ubiquinol-cytochrome c reductase core protein I
Uqcrcl – Ubiquinol-Cytochrome c Reductase Core Protein I
V_m – Membrane potential
X/XO – Xanthine/Xanthine Oxidase
Chapter 1:

Introduction
1.1 Mitochondria, Oxidative Phosphorylation and the Electron Transport Chain:

Mitochondria are membrane bound organelles thought to be derived from an endosymbiotic bacterium (reviewed in Berry, 2003). Principle mitochondrial functions include the production of the majority of cellular ATP via oxidative phosphorylation and provision of a calcium buffering capacity (reviewed in Nicholls & Ferguson, 2002). In addition mitochondria are both a normal physiological source of and a target of reactive oxygen species (ROS) (reviewed in Nicholls & Ferguson, 2002).

ATP is the major molecular energy currency within cells, and powers cellular processes such as biosynthetic reactions, cell motility and cell replication. The production of the majority of the cellular ATP is via the process of oxidative phosphorylation which occurs within mitochondria. Oxidative phosphorylation is the terminal step of respiration in eukaryotic cells whereby reduced co-factors created during glycolysis and the Kreb’s Cycle are used to transfer electrons to the respiratory protein complexes embedded within the inner mitochondrial membrane.

The entry of electrons into the respiratory chain occurs physiologically at two points. NADH transfers electrons to Complex I (NADH dehydrogenase) whilst FADH₂ and succinate transfer electrons to Complex II (succinate dehydrogenase). The two pathways converge at the level of the ubisemiquinone pool, a diffusible carrier which transfers electrons to Complex III (coenzyme Q-cytochrome c oxidoreductase or cytochrome bc₁ complex). Electrons are transferred from Complex III to Complex IV (cytochrome c oxidase) via cytochrome c which is tethered to the inner mitochondrial membrane. Complex IV passes electrons to the terminal electron acceptor – molecular oxygen which is reduced to water (reviewed in Nicholls & Ferguson, 2002). A summary of this mechanism is shown in Figure 1.1.1.

Peter Mitchell proposed in 1961 that the coupling between the electron transfer through the respiratory chain and the synthesis of ATP is indirect (Mitchell, 1961). Overwhelming experimental evidence now supports this theory, and Mitchell was
awarded the Nobel Prize in Chemistry in 1978 for his work. The indirect coupling involves the transport of protons across the inner mitochondrial membrane as electrons are transferred along the electron transport chain thereby producing an electrochemical proton gradient across the inner mitochondrial membrane (low proton concentration within the mitochondrial matrix). This proton pumping activity has been identified to occur at respiratory Complexes I, III and IV (reviewed in (Nicholls & Ferguson, 2002)). This proton gradient may be dissipated through ATP Synthase whilst powering the phosphorylation of ADP to ATP. This mechanism is summarised in Figure 1.1.1. It should also be noted that some protons may passively leak back across the inner mitochondrial membrane thereby dissipating the proton gradient without generating ATP.

![Figure 1.1.1: Schematic diagram of the electron transport chain illustrating the chemiosmotic theory of oxidative phosphorylation as proposed by (Mitchell, 1961).](image)

Rather than undergoing 4 electron reduction to water, some electrons escape the electron transport chain and reduce molecular oxygen to superoxide (reviewed in (Nicholls &
Ferguson, 2002)). This is thought to occur at Complexes I and III of the electron transport chain, although the physiological relevance of superoxide production by Complex III has been questioned (reviewed in (Grivennikova & Vinogradov, 2006)). Superoxide production by the electron transport chain is thought to be the major source of reactive oxygen species under physiological conditions, which may explain the often observed correlation between mitochondrial dysfunction and increased levels of oxidative stress markers (reviewed in (Nicholls & Ferguson, 2002)).

1.2 Mitochondrial Changes in Neurodegeneration:
As brain tissue has the highest oxygen requirement of any body tissue (reviewed in (Halliwell, 2006), it is perhaps unsurprising that mitochondrial dysfunction has been proposed as a contributing factor to many neurodegenerative diseases.

Alzheimer’s Disease is currently the major cause of dementia, with surviving 65 year old humans having a 6.3% and a 10.9% lifetime risk of developing Alzheimer’s Disease for men and women respectively (Seshadri et al., 1997). The disease is associated with the accumulation of intraneuronal fibrillary tangles and neuritic plaques in cortical regions of the brain (reviewed in (Harman, 2006). Experimental evidence indicates mitochondrial structural and functional abnormalities in Alzheimer’s Disease, including detection of mitochondrial morphological abnormalities in cybrid cell lines from Alzheimer’s patients (Trimmer et al., 2000), reduced activity of Complex IV of the mitochondrial Electron Transport Chain (ETC) (Parker et al., 1990; Kish et al., 1992; Bosetti et al., 2002), increased rates of mitochondrial DNA mutation in brain tissue from Alzheimer’s sufferers (Coskun et al., 2004) and altered expression of mitochondrial electron transport complex genes (Manczak et al., 2004).

Parkinson’s Disease is a neurodegenerative condition affecting around 1% of the population by age 70 and involves the degeneration of the substantia nigra and accumulation of Lewy bodies in brain tissue (reviewed in (Savitt et al., 2006). Mitochondrial morphological abnormalities have been observed in Parkinson’s Disease
(Trimmer et al., 2000), and the disease is strongly associated with a loss of activity of mitochondrial Electron Transport Chain Complex I (Schapira et al., 1990), possibly due to oxidative damage of its subunits (Keeney et al., 2006). Additionally mutation of the PINK1 gene, a mitochondrially localized kinase, has been associated with some familial cases of early onset Parkinson’s Disease (Valente et al., 2004).

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder affecting the voluntary motor neurons and has an incidence of approximately 1 or 2 per 100,000 of the population (Roman, 1996). Mitochondrial involvement in the condition has been indicated by abnormalities in mitochondrial structure (Sasaki et al., 2004) and alterations in enzymatic activities of the respiratory chain, although a number of reports present conflicting data regarding the nature of these alterations (Bowling et al., 1993; Wiedemann et al., 1998; Browne et al., 1998). Mitochondrial dysfunction has been implicated in neuronal death in ALS (Hervias et al., 2006) due to the key roles mitochondria play in apoptosis (Green & Kroemer, 2004).

It therefore appears that abnormalities in mitochondrial morphology and electron transport chain activity are common features of neurodegenerative disease.

1.3 Protein misfolding in neurodegeneration:
Another hallmark of many neurodegenerative conditions is the deposition of abnormally folded and oxidized protein within brain tissue. Alzheimer’s Disease involves deposition of β-amyloid and tau protein, Parkinson’s Disease involves α-synuclein deposition and amyotrophic lateral sclerosis involves tau protein deposition (reviewed in Carrell & Lomas, 1997). Aggregation of these proteins to form insoluble fibrils results in cellular damage, and aggregate accumulation has been demonstrated to correlate with evidence of neuropathology (reviewed in Sipe & Cohen, 2000). Research into the neurotoxic qualities of these protein aggregates is ongoing, but it has been suggested that they cause cellular damage via generation of free radicals by binding metal ions (Halliwell, 2006), by mediating mitochondrial damage (Halliwell, 2006) and by physical...
blocking of axonal transport which results in the degeneration of nerve projections (Roy et al., 2005). The transmission of neurodegenerative protein misfolding disorders may be through familial, sporadic or infectious mechanisms (reviewed in (Soto et al., 2006).

1.4 Oxidative Stress in Neurodegeneration:

The concept of oxidative stress was formally defined in 1991 by Sies as an ‘imbalance in this pro-oxidant anti-oxidant equilibrium in favor of the pro-oxidants’ (Sies, 1991). It therefore follows that either increased rates of oxidant formation or compromised antioxidant defence can lead to a state of oxidative stress. Oxidative stress can lead to the oxidative modification of many cellular macromolecules, including lipids, proteins and DNA (reviewed in (Halliwell & Gutteridge, 1999), thereby compromising their functionality and resulting in cellular damage.

The major physiological source of reactive oxygen species (ROS) is thought to be leakage of superoxide radicals from the mitochondrial electron transport chain (reviewed in (Raha & Robinson, 2000). Whilst superoxide itself is relatively unreactive towards biological macromolecules, superoxide may be converted to more reactive species such as the hydroxyl radical or peroxynitrite anion which can directly damage cellular macromolecules. To prevent this potential damage, organisms possess a myriad of antioxidant defences which can detoxify reactive oxygen species.

The brain is an organ which is particularly susceptible to oxidative damage due to its high levels of oxygen consumption, the presence of auto-oxidizable neurotransmitters, the presence of excitatory amino acid neurotransmitters, relatively high iron concentrations, high levels of polyunsaturated fatty acids in lipids which provide targets for lipid peroxidation, production of ROS via normal brain metabolism and low levels of antioxidant defence (reviewed in (Halliwell, 2006). It is therefore unsurprising that oxidative stress is through to participate in a number of neurodegenerative conditions.
Typically evidence of oxidative damage to cellular macromolecules is taken to indicate the involvement of oxidative stress in a neurodegenerative condition. Markers of oxidative damage commonly assessed include end-products of lipid peroxidation, evidence of protein oxidation/nitration and evidence of DNA oxidative modification. Increased levels of such marker compounds have been detected in a number of neurodegenerative conditions. Studies of Alzheimer’s Disease have uncovered increased levels of protein carbonyls (Hensley et al., 1995), 4-hydroxynonenal (Ando et al., 1998) and 3-nitrotyrosine (Hensley et al., 1998). Studies of Parkinson’s Disease have detected increased levels of protein carbonyls (Alam et al., 1997), 3-nitrotyrosine (Good et al., 1998) and nitrated α-synuclein (Giasson et al., 2000). Studies of ALS have detected increased levels of protein carbonyls (Ferrante et al., 1997), 3-nitrotyrosine (Beal et al., 1997) and oxidized DNA (Bogdanov et al., 2000). The hypothesis that oxidative stress plays an integral role with the pathogenesis of these conditions is further strengthened by many of these studies indicating a correlation between increased levels of markers of oxidative stress and evidence of neurodegeneration.

Compelling evidence has accumulated implicating protein aggregation, mitochondrial dysfunction and oxidative stress in the pathogenesis of many neurodegenerative diseases. A link between these events has been suggested whereby oxidative stress results in damage to cellular macromolecules and mitochondria, dysfunctional mitochondria produce larger quantities of free radicals, and abnormally folded proteins are formed due to oxidative stress and go on to produce free radicals themselves (Halliwell, 2006). Therefore despite different neurodegenerative diseases targeting different specific brain regions, a common mechanism(s) may be involved in the pathogenesis of many of these conditions.

1.5 Transmissible Spongiform Encephalopathies:
Transmissible Spongiform Encephalopathies (TSE’s) are neurodegenerative conditions including the human diseases Creutzfeldt-Jakob Disease (CJD), Fatal Familial Insomnia (FFI), Fatal Sporadic Insomnia (FSI), Gerstmann-Sträussler-Scheinker Disease (GSS)
and Kuru, as well as a number of conditions that affect animals including Scrapie in sheep, Bovine Spongiform Encephalopathy (BSE) in cattle, Transmissible Mink Encephalopathy (TME) in mink and Feline Spongiform Encephalopathy (FSE) in cats (Budka, 2003).

The pathological hallmarks of these neurodegenerative conditions include a loss of neurons, spongiform degeneration of the CNS and gliosis (propagation of astroglia and microglia) eventually leading to death (reviewed in (Budka, 2003). Aggregates of abnormally folded prion protein are also evident in the brain tissue of TSE suffering organisms, the pattern of deposition of which varies between TSE’s (reviewed in (Budka, 2003). Mitochondrial involvement in TSE’s was initially suggested due to the association of mitochondrial nucleic acids with detergent insoluble membrane fractions, and the high degree of scrapie infectivity contained within the mitochondrial fraction of scrapie infected hamster brain (Aiken et al., 1989). Subsequently abnormalities in mitochondrial structure have been identified following scrapie inoculation (Jeffrey et al., 1991; Choi et al., 1998; Lee et al., 1999) and in GSS (Liberski & Budka, 1995), and altered activity of the respiratory chain (Choi et al., 1998). Evidence pointing to increased levels of oxidative stress in TSE’s includes increased levels of protein nitration and carbonyl formation (Wong et al., 2001a) and increased lipid peroxidation (Kim et al., 2000; Wong et al., 2001a) in scrapie infected mice. Indeed mitochondria have been suggested to be a source of increased superoxide radical production following scrapie inoculation (Lee et al., 1999), and increased levels of multimeric mitochondrial DNA have been detected in the brain tissue of scrapie infected hamsters (Narang et al., 1991).

Therefore, as with many other neurodegenerative diseases, TSE’s show evidence of abnormal protein accumulation, mitochondrial dysfunction and oxidative stress.
1.6 Identification of the Prion Protein:

Initial steps towards uncovering the nature of the infective agent in TSE’s involved enrichment of brain fractions from Syrian Golden Hamster and resulted in the discovery of the PrP 27-30 prion protein fragment (Bolton et al., 1982; Prusiner et al., 1982). Determination of the amino terminal sequence of the prion protein (PrP) (Prusiner et al., 1984) permitted later molecular cloning studies to be performed to elucidate the sequence of the prion protein (Oesch et al., 1985) and the discovery that the endogenous protein PrPc and PrPSc were encoded by the same gene (Basler et al., 1986).

The discovery of the nature of PrPc and PrPSc lead to the prion (protein-only) hypothesis of transmissible spongiform encephalopathy infectivity being put forward by Prusiner (Prusiner, 1982). A large body of evidence now supports this hypothesis including that the infectious agent is too small to be a micro-organism (Alper et al., 1966), infectivity is maintained following procedures that normally destroy nucleic acids (Alper et al., 1967), that the inactivation of PrPSc attenuates infectivity (Prusiner et al., 1993b), that PrPc-null animals fail to develop TSE following inoculation with scrapie (Bueler et al., 1993), that PrPc can be converted to PrPSc in an auto-catalytic fashion (Kocisko et al., 1994) and because it has not been possible to consistently detect any nucleic acid associated with the infectious agent (Safar et al., 2005).

The prion protein is expressed endogenously in a form designated PrPc. This form of the protein is mainly α-helical in structure, but may undergo a pathogenic conformational change into a predominantly β-sheet version designated PrPSc (Pan et al., 1993). PrPSc is protease and detergent resistant (Caughy and Raymond 1991) and forms deposits in the brains of TSE suffering organisms. PrPSc itself has been proposed as the infectious agent in prion diseases, acting either as a seed or template for the conversion of endogenous PrPc to PrPSc (Jarrett & Lansbury, 1993). It has been determined that maximal infectivity per unit mass of PrPSc occurs when PrPSc is aggregated to form oligomers of 300-600kDa and that infectivity is reduced for large fibrils and even more
so for small oligomers of PrPSc, indicating the importance PrPSc aggregate formation in its replication process (Silveira et al., 2005).

In humans a number of different mechanisms have been shown to result in the development of TSE’s. These include germline mutations in the PrP gene (familial CJD, Fatal Familial Insomnia, GSS), spontaneous mutation of the PrP gene or spontaneous conversion of PrPc to PrPSc (sporadic CJD), medical exposure to CJD contaminated tissue (iatrogenic CJD) or ingestion of CJD contaminated food (variant CJD) (Prusiner, 1998). In the case of vCJD PrPSc has been shown to travel to the nervous system via the lymphoid system (Mabbott & MacPherson, 2006).

Currently the major aims of research into prion diseases are the study of transmissibility, study of PrPSc formation and its effects, and determination of the normal physiological function of PrPc.

1.7 Tissue and cellular distribution of PrPc:

The prion protein is expressed in many tissues throughout the body, including the brain, skeletal muscle, lung, heart, intestinal tract and spleen amongst other organs (Bendheim et al., 1992). PrPc levels are particularly high in the brain where expression occurs within both neuronal (Kretzschmar et al., 1986) and non-neuronal cells (Moser et al., 1995). PrPc distribution within the brain has been determined using electron microscopy and immunohistochemical techniques, revealing expression of high concentrations of PrPc in the striatonigral complex and the hippocampus and more moderate levels of expression within the cortex and the olfactory system (Sales et al., 1998;Herms et al., 1999). PrPc attaches to the cell membrane via a glycosylphosphatidylinisotol (GPI) anchor found at its C-terminal (Stahl et al., 1987), and has been found to exist at high concentrations at synapses (Fournier et al., 1995;Moya et al., 2000). This synaptic distribution of PrPc, which is particularly prevalent within areas known for ongoing plasticity (Moya et al., 2000), suggests that prion protein may have a role in synaptic plasticity.
Whilst TSE's involve the conversion of PrP\textsuperscript{C} to its pathogenic isoform PrP\textsuperscript{Sc}, it has yet to be ascertained whether prion diseases result from the loss of an endogenous PrP\textsuperscript{C} function, a toxic gain of function by PrP\textsuperscript{Sc}, or a combination of the two. The issue of whether PrP\textsuperscript{C} loss contributes to TSE pathogenesis is hindered by the fact that a normal physiological function of PrP\textsuperscript{C} has yet to be agreed upon. However the PrP gene is highly conserved across species (Westaway & Prusiner, 1986), and shows high levels of amino acid sequence identity (92.9 and 99.6\%) between primates and humans (Schatzl et al., 1995), which suggests that PrP\textsuperscript{C} carries out an essential cellular function.

1.8 Toxic gain of function by PrP\textsuperscript{Sc}:
PrP\textsuperscript{Sc} accumulation in the brain correlates both spatially (to a cellular level) and temporally with neuropathology (Jeffrey et al., 2001). This suggests that a toxic gain of function by PrP\textsuperscript{Sc} may be responsible for neuronal death in TSE's. The lack of an overt phenotype in PrP-null mice (Bueler et al., 1992; Manson et al., 1994) supports this hypothesis.

However it is uncertain whether the infectious agent PrP\textsuperscript{Sc} is itself toxic or whether the correlation between PrP\textsuperscript{Sc} generation and neuropathology is epiphenomenal. The case for PrP\textsuperscript{Sc} accumulation causing neurodegeneration is challenged by a number of conditions where neuronal death occurs with low levels of or no PrP\textsuperscript{Sc} accumulation, including a number of familial TSE's such as GSS (Hegde et al., 1998), and the lack of PrP\textsuperscript{Sc} accumulation in over 55\% of C57BL/6 mice inoculated with BSE prions despite neuronal death occurring (Lasmezas et al., 1997). Similarly the presence of PrP\textsuperscript{Sc} within a brain region has been shown to not necessarily result in neuropathology in that region (Hayward et al., 1994), and it has been shown that mice inoculated with hamster scrapie accumulate PrP\textsuperscript{Sc} without showing clinical symptoms of the disease (Hill et al., 2000).
1.9 Transgenic models to study the effects of PrP\(^C\) loss:

A number of transgenic mouse models lacking PrP\(^C\) expression have been created. These models provide potential systems in which to study the normal physiological function(s) of PrP\(^C\).

The first PrP\(^C\)-null mouse, the Zurich Mouse, was produced in 1992 (Bueeler et al., 1992). Two years later another PrP\(^C\)-null mouse, the Edinburgh Mouse, was created using a different gene targeting strategy (Manson et al., 1994). Neither of these mouse models showed any overt phenotypic differences from control mice in behavioural tests (Bueeler et al., 1992; Manson et al., 1994). The third and fourth PrP\(^C\)-null models (Sakaguchi et al., 1996; Moore et al., 1999) both exhibited a similar late onset fatal ataxia which became evident after approximately 70 weeks. This phenotype was not evident in either the Zurich or Edinburgh mouse models. However studies showed that this phenotype was due to an overexpression of prion-like protein Doppel due to accidental removal of a control sequence (Moore et al., 1999). Doppel has been demonstrated to be functionally antagonistic to PrP (Qin et al., 2006).

The production of these PrP-null lines of mice furthered the case for the prion protein playing an integral role in the development of TSE’s, as the PrP-null strains of mouse proved resistant to scrapie infection (Bueeler et al., 1992; Prusiner et al., 1993a). However these studies suggested that the loss of PrP\(^C\) could not be definitively linked to neurodegeneration in TSE’s, and because it was also not possible to link PrP\(^\text{Sc}\) accumulation to neurodegeneration, it was postulated that a third form of the prion protein existed which was neurotoxic and was generated at the same time as PrP\(^\text{Sc}\) (Chiesa & Harris, 2001).

In order to further lines of research regarding the functions of PrP\(^C\), this study will utilize the Edinburgh PrP-null mouse (Manson et al., 1994).
1.10 Studies of PrP-null Mice:
The Zurich and Edinburgh strains of PrP-null mice initially displayed no overt phenotype (Bueler et al., 1992; Manson et al., 1994), although abnormalities in sleep patterns were observed in the Edinburgh strain. The disturbed sleep patterns observed in the Edinburgh strain of PrP-null mouse (Tobler et al., 1997) have resonance with the fact that sleep abnormalities accompanied by mutation of PrP are observed in FFI (Montagna et al., 2003), and indeed sleep abnormalities have recently been detected in humans suffering from sCJD (Landolt et al., 2006).

Despite the lack of an overt phenotype for the Zurich or Edinburgh PrP-null mice relative to their appropriate controls, a number of studies have revealed some subtle, and sometimes controversial, differences.

Electrophysiological studies of PrP-null mice have centred upon the hippocampus, a region which shows synaptic plasticity and which also normally synaptically expresses PrPc at high levels (Sales et al., 1998). Initial electrophysiological studies of PrP-null animals revealed that the nulls have reduced GABA-ergic mediated inhibition and reduced long-term potentiation (Collinge et al., 1994). Reduction in the after-hyperpolarisation current Iahp has been observed in PrP-null mice (Colling et al., 1996). Rescue of the PrP-null electrophysiological phenotype has been demonstrated by introduction of human E200K PrP into mice (Asante et al., 2004), indicating that the phenotype observed in PrP-null mice is due to a loss of prion protein.

However other groups have disputed these findings. Lledo et al reported no alteration in GABAergic transmission or the Iahp in PrP-null mice (Lledo et al., 1996). More recently it has been reported that PrPc expression is involved in the facilitation of synaptic transmission in the Zurich mouse (Carleton et al., 2001), and reductions in post-tetanic potentiation (PTP) and in long-term potentiation (LTP) have been found to develop with age in the Edinburgh strain of PrP-null mice (Curtis et al., 2003).
A Cre-recombinase loxP system has been used to produce mice that can have their expression of PrP\textsuperscript{C} inactivated at the adult stage, thus allowing the study of PrP\textsuperscript{C} effects without the possibility of compensation for the loss of PrP\textsuperscript{C} or adverse effects due to variable genetic backgrounds (Mallucci et al., 2002). Whilst no alterations in the input resistance, resting membrane potential, action potential threshold or action potential amplitude were detected, these mice showed a significant reduction in the I\textsubscript{AHP} following inactivation of PrP\textsuperscript{C} (Mallucci et al., 2002). This suggests that the alteration in the I\textsubscript{AHP} is due to a modulatory effect of PrP\textsuperscript{C} and not a compensation for loss of PrP\textsuperscript{C}.

I\textsubscript{AHP} and PTP are linked by the fact that both are controlled by mitochondrial calcium buffering. I\textsubscript{AHP} is mediated by a calcium-activated potassium current (Gustafsson & Wigstrom, 1981) which is potentially subject to modification by mitochondrial calcium buffering. PTP is controlled by the release of calcium ions from mitochondria which have accumulated during tetanic stimulation, this elevates the intracellular calcium ion concentration within the nerve terminal and increases neuronal excitability (Tang & Zucker, 1997; Zucker & Regehr, 2002). It is therefore possible that a mitochondrial abnormality could underlie these electrophysiological abnormalities in PrP-null mice.

The case for mitochondrial abnormality in PrP-null mice was further strengthened by a study of gene expression conducted by Miele et al. Studies of gene expression by Differential-Display-Reverse-Transcriptase polymerase chain reaction (DDRT PCR) in the Edinburgh PrP-null mouse revealed altered expression of a number of genes, including the nuclear encoded genes for two fragments of PrP itself and alteration in transcription of a subunit of respiratory Complex I, along with the mitochondrially encoded genes for a subunit of respiratory Complex IV and 16S rRNA (Miele et al., 2002). Whilst both alterations were subtle, the directionality of the changes in respiratory chain subunit transcription were an upregulation of Complex I subunit NADH ubiquinone oxidoreductase B14.5b and a downregulation of Complex IV cytochrome oxidase subunit I (Miele, 2000). Furthermore mitochondrial morphological abnormalities in the Edinburgh PrP-null mouse were identified by Miele et al by
morphometric analysis of the CA1 neuropil region of the hippocampus studied under electron microscopy. Mitochondrial number was found to be reduced in PrP-null animals, whilst those mitochondria present were increased in diameter and a greater proportion appeared abnormal showing ‘sparse and poorly defined cristae’ (Miele et al., 2002).

Three previous studies have investigated respiration rates in PrP-null mice. The respiration rates of cardiac mitochondria were found to be unaltered in the Edinburgh PrP-null mouse (Miele et al., 2002). The activities of Complexes I and II/III were found to be unaltered in brain homogenate from the Edinburgh PrP-null mouse (Brown et al., 2002). And finally the respiration rates of mitochondria isolated from a variety of brain regions from the Zurich PrP-null mouse have been reported to be unaltered upon provision of succinate as a respiratory substrate (Lobao-Soares et al., 2005). Whilst these studies may suggest that mitochondrial respiration is unchanged in PrP-null mice, the study of Miele et al was carried out using a non-neuronal tissue, Brown et al utilized crude brain homogenate rather than isolated mitochondria which could result in glutathionylation of Complex I (Taylor et al., 2003) thereby reducing its activity, and the study of Lobao-Soares et al failed to study the respiratory chain in its entirety. Therefore scope remains for there to be abnormalities in mitochondrial respiration in PrP-null mouse brain.

This study will attempt to further identify mitochondrial abnormality(s) in mitochondria isolated from brain tissue of the Edinburgh PrP-null mouse.

1.11 Putative functions of PrP<sup>C</sup>:

Whilst a gain of a toxic property by PrP<sup>Sc</sup> or another abnormal isoform of the endogenous prion protein may cause the neurodegeneration observed in TSE’s, it is also possible that the diseases result from a loss of the normal physiological function of PrP<sup>C</sup>. Whilst consensus regarding the normal physiological function of PrP<sup>C</sup> has yet to be
reached, the majority of experimental evidence suggests the PrP<sup>C</sup> is neuroprotective via a number of different mechanisms.

The endogenous prion protein PrP<sup>C</sup> possesses a highly conserved octapeptide repeat region towards its N-terminal which has been identified as being capable of binding Cu<sup>2+</sup> ions (Hornshaw et al., 1995), and indeed it was later determined using PrP-null mouse models that PrP<sup>C</sup> binds copper in vivo (Brown et al., 1997a). As copper ions play integral roles within a number of different enzymes, whilst also being capable of catalysing damaging free radical reactions, the importance of appropriate copper transporting systems can be appreciated. A role for PrP<sup>C</sup> in the import of copper ions has been proposed as PrP-null animals have been found to possess considerably reduced copper concentrations in membrane-rich brain extracts when compared to control animals (Brown et al., 1997a). Another study furthered the case for PrP<sup>C</sup> involvement in copper uptake when it was found that the presence of copper promotes the endocytosis of PrP<sup>C</sup> from the plasma membrane, thereby facilitating copper uptake (Pauly & Harris, 1998). A role for PrP<sup>C</sup> in metal transport is further strengthened by the fact that concentrations of a number of divalent metal ions (including Cu<sup>2+</sup>, Fe<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup> and Ca<sup>2+</sup>) are altered in scrapie (Wong et al., 2001a).

The binding of copper ions to PrP<sup>C</sup> has also been demonstrated to confer a SOD-activity to the protein (Brown et al., 1999; Wong et al., 2000), although this finding has been disputed by others (Waggoner et al., 2000; Hutter et al., 2003). This property would suggest that the endogenous prion protein plays a role as an antioxidant. The localization of PrP<sup>C</sup> to the synapse (Sales et al., 1998) could indicate that PrP-SOD activity is a major or the major antioxidant species in this region. Additionally the ability of PrP<sup>C</sup> to bind potentially toxic copper ions may provide an additional neuroprotective property (Brown et al., 1997a). The loss of these neuroprotective qualities localized to the synapse could therefore indicate why electrophysiological abnormalities and a loss of synapses are detectable during the onset of scrapie (Clinton et al., 1993; Johnston et al., 1998). Furthermore the case for PrP<sup>C</sup> carrying out an
antioxidant function is strengthened by the finding that PrP-null mice suffer from increased levels of oxidative stress (Wong et al., 2001b), and because expression of PrP<sup>C</sup> in cultured rabbit kidney epithelial cells has been demonstrated to reduce markers of oxidative damage and reduce cell death following exposure to paraquat (Senator et al., 2004).

Some studies have implicated this copper binding property of PrP<sup>C</sup> as being involved in the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup>. Evidence supporting this hypothesis includes that the binding of copper to PrP<sup>C</sup> results in the formation of an isoform of the protein which exhibits greater protease and detergent resistance but is distinct from PrP<sup>Sc</sup> (Quaglio et al., 2001), that addition of a copper chelating compound delays the onset of prion disease (Sigurdsson et al., 2003) and that circular dichroism analysis revealed that the binding of copper to a fifth site induced the formation of a β-sheet formation as is observed in PrP<sup>Sc</sup> (Jones et al., 2004). Therefore whilst copper may play an integral role in the endogenous function of PrP<sup>C</sup>, it may also be involved in the pathogenic conformational changes of PrP<sup>C</sup> involved in TSE's.

In addition to the interaction between PrP and copper, numerous other interactions have been characterised in neurons, these are summarised in Table 1.11.1.

Experimental therefore supports that PrP<sup>C</sup> participates within a diverse array of cellular processes. Whilst some of these reports dispute each others findings, considerable amounts of the published evidence points to PrP<sup>C</sup> being neuroprotective. It is therefore the loss of a physiological function of PrP<sup>C</sup> may cause or be involved in the pathogenesis of TSE's.
Table 1.11.1: Binding partners of PrP:

<table>
<thead>
<tr>
<th>Binding Partner</th>
<th>Function</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>STI1 (Zanata et al., 2002)</td>
<td>Neuroprotective</td>
<td>STI1 binding activates Cu Zn SOD activity (Sakudo et al., 2005b) via PKA pathway (Lopes et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Neuritogenesis</td>
<td>STI1 binding activates mitogen-activated protein kinase causing neuritogenesis (Lopes et al., 2005)</td>
</tr>
<tr>
<td>Laminin (Graner et al., 2000)</td>
<td>Neuritogenesis</td>
<td>Interaction between PrPSc and laminin involved in nerve growth factor mediated neuritogenesis (Graner et al., 2000)</td>
</tr>
<tr>
<td>NCAM (Santuccione et al., 2005)</td>
<td>Neuritogenesis</td>
<td>Promotes NCAM recruitment into lipid rafts resulting in neuritogenesis (Santuccione et al., 2005)</td>
</tr>
<tr>
<td>LRP/LR (Morel et al., 2005)</td>
<td>Internalization</td>
<td>Binding of PrP to the laminin receptor stimulates endocytosis in enterocytes (Morel et al., 2005)</td>
</tr>
<tr>
<td>Apolipoprotein E (Baumann et al., 2000)</td>
<td>Amyloidogenic</td>
<td>Accelerates formation of amyloid fibrils (Baumann et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Pro-apoptotic</td>
<td>Induces the mitochondrial apoptotic pathway (Mahley et al., 2006)</td>
</tr>
<tr>
<td>Bel-2 (Kuwahara et al., 1999)</td>
<td>Anti-apoptotic</td>
<td>Anti-apoptotic via a Bel-2 like inhibition of Bax (Bounhar et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Pro-apoptotic</td>
<td>PrPSc co-aggregates with Bel-2 promoting Bax mediated apoptosis (Rambold et al., 2006)</td>
</tr>
<tr>
<td>GFAP (Oesch et al., 1990)</td>
<td>?</td>
<td>GFAP knockout mice develop TSE’s indicating interaction unimportant in disease development (Gomi et al., 1995)</td>
</tr>
</tbody>
</table>

1.12 Evidence of oxidative stress in PrP-null mice:
In addition to evidence of oxidative stress in TSE’s, data has also been published indicating increased levels of oxidative stress in PrP-null mice. The study of cultured PrP-null cerebellar neurons has repeatedly revealed that they are more susceptible to cell death than wild-type controls, especially under pro-oxidative conditions (Brown et al., 1997b; Brown et al., 1998; Brown et al., 2002). Interestingly the increased sensitivity to oxidative stress is limited to neurons and does not affect glial cells (Brown et al., 1998).
This susceptibility to cell death has been suggested to be due to sensitivity to oxidative stress. Evidence to support this hypothesis includes that PrP-null neurons can be protected from degeneration by the addition of the antioxidant vitamin E into the culture medium (Brown et al., 1997b), and by addition of the octa-repeat region of PrPc (Brown et al., 1998) which has been suggested to possess antioxidant SOD activity (Brown et al., 1999). A more recent study has also indicated that PrP-null neuronal cells are intrinsically more susceptible to cell death following serum (and therefore antioxidant) withdrawal in cell culture systems, suggesting an increased susceptibility to oxidative stress (Kim et al., 2004).

Increased levels of markers of oxidative damage to proteins and lipids in PrP-null mice have been detected (Wong et al., 2001b) indicating increased levels of damage to cellular macromolecules which is indicative of a state of oxidative stress.

Various molecules that participates within the cellular antioxidant defenses have also been demonstrated to show altered activity in PrP-null mice (Brown et al., 2002) which further suggests a state of oxidative stress. It has been hypothesized that PrPc may act as a signaling molecule which acts to regulate the cellular antioxidant defense (Brown, 2005) which would provide an explanation as to why so many antioxidant systems are affected.

Whilst considerable evidence has accumulated indicating that PrP-null neurons are susceptible of to oxidative stress (Brown et al., 1997b; Brown et al., 1998; Kim et al., 2004) and possess altered levels of cellular antioxidants (Brown et al., 2002), it remains unresolved whether oxidative damage is due solely to a loss of antioxidants, or whether increased production of free radicals also plays a role. As mitochondria have been reported to be abnormal in PrP-null mice (Miele et al., 2002) and are a major source of free radicals, it is possible that increased mitochondrial superoxide production may contribute towards the oxidative damage observed in PrP-null mice (Wong et al., 2001b).
1.13 Measurement of oxidative stress:
Mitochondria are the major physiological source of reactive oxygen species within cells, where the partial reduction of molecular oxygen by redox centres of the respiratory chain releases superoxide radicals (Raha & Robinson, 2000). It is therefore of interest to be able to measure the rates of superoxide radical production by the respiratory chain.

As previously described, oxidative stress involves the balance between pro- and antioxidant systems. Steady state concentrations of superoxide radicals are usually kept at very low levels by antioxidant systems, and it has been estimated that cellular superoxide concentrations are normally around 1nM (Tarpey et al., 2004). However chronic exposure to oxidative stress, rather than an acute oxidative insult, is thought to underlie many neurodegenerative conditions, and therefore small increases in steady state free radical concentrations may lead to exacerbated levels of oxidative damage and neuronal death (Beal, 1998). However such small increases in superoxide formation rates are technically difficult to assess. A variety of different assays for oxidative stress have been characterised in the literature.

A potential assay for oxidative stress in biological systems is to assess a biological response to oxidative stress. Commonly used assays include determination of cell death rates and loss of contractile response of smooth muscle cells in response to oxidative insult. However whilst such studies may be sensitive to oxidative stress, net oxidative damage rather than free radical production is often detected, such assays rely on the availability of a suitable biological response, tissue responses may not vary directly with oxidative stress, and such assays require considerable validation to ensure that the observed effect is due to oxidative stress.

Oxidative stress results in oxidative damage to various cellular components including lipids, protein and DNA (reviewed in Halliwell, 2006). Many techniques for assaying oxidative stress involve the detection of stable end products of oxidative damage to cellular macromolecules which accumulate over time. Such assays include detecting
malondialdehyde (MDA) as an indicator of lipid peroxidation, detection of 8-hydroxyguanine as an indicator of DNA oxidation or the assay of protein carbonyl groups indicating protein oxidation (reviewed in Griffiths et al., 2002). However, these techniques detect the end-products of oxidative damage and are a product of free radical production rates, level of antioxidant protection and the availability of the relevant macromolecular target. Whilst these measures may provide a good assessment of the oxidative state of a tissue, the source of the oxidative damage is not necessarily obvious.

An alternative to assaying markers of oxidative stress is the direct assessment of free radical concentrations. The spectrophotometric determination of ferricytochrome c reduction has long been a commonly used assay for the superoxide radical (Fridovich, 1970). However, this assay lacks sensitivity, selectivity, is susceptible to interference by antioxidants, and ferricytochrome c has limited intracellular access.

A variety of different fluorophores have been developed to allow the detection of reactive oxygen species thereby providing opportunities to assay free radical production both spatially and temporally. Particular problems associated with the use of fluorescent detection of reactive oxygen species are auto-oxidation of fluorescent probes resulting in background fluorescence and a lack of specificity of some fluorescent probes for particular reactive oxygen species (reviewed in Soh, 2006). However, many novel probes for reactive oxygen species, including superoxide selective probes, have recently been characterised which may aid the future study of reactive oxygen species in biology using fluorescent techniques (reviewed in Soh, 2006).

Recently, the technique of electron paramagnetic resonance (EPR) spectroscopy has been applied to the measurement of free radical concentrations in biological samples.

1.14 Electron paramagnetic resonance spectroscopy and the detection of free radicals: EPR spectroscopy is capable of directly detecting compounds with unpaired electron(s), and therefore is capable of detecting free radicals. Normally the 2 spin states of
unpaired electrons are degenerate. However within a magnetic field, electron spin aligns either parallel or anti-parallel to the magnetic field thereby creating distinct energy levels. Absorbance of microwave energy permits the transition of an unpaired electron between these two spin states. To measure free radical concentrations within a sample, the sample is placed within a microwave beam and subjected to varying intensities of magnetic field strength. Microwaves are absorbed when a state of resonance is reached i.e. when quanta of microwaves possess the correct amount of energy required to flip an electron between spin states. Microwave absorbance is therefore proportional to free radical concentration.

However the low steady state concentrations of free radicals found physiologically makes their direct detection using EPR impossible at physiological temperature. The advent of spin trapping compounds finally permitted the application of EPR spectroscopy to the detection of free radicals in biological samples (reviewed in Kopani et al., 2006). Spin traps are compounds capable of reacting with free radicals, and the trapping of a radical results in the production of a radical compound with greater stability than the parent radical. Various spin trapping compounds have been assessed for use in biological applications, each with their inherent benefits and limitations.

This study will primarily employ hydroxylamine spin traps, and in particular tempone-H. This spin trapping compound can undergo hydrogen abstraction via reaction with superoxide radicals or peroxynitrite to form a stable free radical known as tempone which can be detected by EPR spectroscopy (Dikalov et al., 1997a).

![Chemical structure of Tempone-H (A) and Tempone (B).](image-url)
The chemical structures in Figure 1.14.1 demonstrates how tempone-H (Panel A) may be oxidized by some species to tempone (Panel B). The reactivity of the unpaired electron of tempone is low due to shielding of this centre by the four methyl groups.

However this hydrogen abstraction process of spin trapping results in the loss of information regarding the identity of the parent radical. Therefore to identify the radical responsible for tempone-H oxidation it is necessary to carry out additional experiments. This is in contrast to some older spin traps such as DMPO, DEPMPO and TMIO which form chemically distinct spin trap adducts with distinct EPR spectra according to the radical species trapped.

EPR spectra are displayed as the first derivative of the absorption spectrum, and usually consist of a number of peaks. In the case of tempone, the EPR spectrum consists of three distinct peaks. This is due to modulation of the energy difference between electron spin states by nuclear spin. In the case of Tempone, the nuclear spin of the nitrogen nucleus splits the spectrum into 3 equal peaks.

The main advantage of tempone-H over older spin traps such as DMPO is firstly that it has a rate constant with superoxide radicals approximately 10-times greater than that of DMPO and superoxide (Dikalov et al., 1997a), and secondly tempone (unlike DMPO spin trap adducts) is stable in solution and therefore may accumulate. However tempone-H is subject to auto-oxidation and therefore background EPR signal accumulates in the absence of free radical production. In addition cellular antioxidant defenses compete with the spin trap and may therefore control the measured free radical concentrations. Therefore there is considerable potential for antioxidant systems to interact with tempone-H spin trapping.
This thesis will attempt to characterize tempone-H as a spin trapping agent with the aim of developing a protocol to assess superoxide formation from the respiratory chain of mitochondria.

1.15 Aims of the study of PrP-null mice:
Considerable experimental evidence indicates mitochondrial structural and functional abnormalities and oxidative stress in TSE’s. This thesis sets out to further lines of evidence which suggest increased levels of oxidative stress and mitochondrial impairment in PrP-null mice. Evidence of an oxidative stress-related phenotype in PrP-null mice will be sought by electrophysiological measurement of synaptic efficacy and determination of neuronal survivability in organotypic cell culture of the hippocampus. The case of whether mitochondria in PrP-null mouse brain are structurally abnormal will be revisited by assessment of mitochondrial morphology using electron microscopy, and confirmation of abnormalities in SOD activity in PrP-null mouse brain will be sought using a spectrophotometric assay of SOD activity. Whilst published evidence suggests that mitochondrial respiration is unaltered in PrP-null mice, respiration was either measured in non-neuronal tissues or the analysis of respiration rates did not cover the complete respiratory chain. To definitively ascertain whether mitochondrial respiration is altered in PrP-null mouse brain, respiration rates of isolated brain mitochondria will be measured across the entire respiratory chain. Whilst evidence of oxidative stress and evidence of reduced antioxidant protection has been detected in PrP-null mice, no study has determined whether increased production of superoxide by mitochondria contributes to the detected oxidative damage. The final part of this study will attempt to develop a technique to allow an assessment of superoxide radical production by the respiratory chain using EPR, the results of which may help disambiguate whether the increased levels of oxidative damage markers are due to a loss of antioxidant protection alone, or due to a loss of antioxidant protection in conjunction with increased superoxide production by the respiratory chain.
1.16 Rett Syndrome:
During the study of mitochondrial function in PrP-null mice, the opportunity arose to participate in a collaborative study of mitochondria in MeCP2-null mice, a mouse model of Rett Syndrome. In addition to being of general scientific interest, the comparison of results from this study to those from PrP-null mice provides information regarding the specificity of any mitochondrial abnormalities to the mouse model under investigation.

Rett Syndrome, a condition first described by Andreas Rett in 1966 (Rett, 1966) is a neurodevelopmental disorder which affects mainly females, is predominantly sporadic and has an incidence of approximately 1 in 10,000 to 22,000 female births (Kriaucionis & Bird, 2003). Sufferers of Rett syndrome develop normally for a variable period of time, typically 6-18 months, before the progressive onset of symptoms which include loss of language, motor and cognitive skills and social withdrawal (Williamson & Christodoulou, 2006).

Classical Rett Syndrome is a condition in humans that has been found to be caused by mutation of the methyl-CpG-binding protein 2 gene (MECP2) (Amir et al., 1999), such mutations being detectable in 90-95% of sufferers (Williamson & Christodoulou, 2006). MeCP2 protein functions as a methylation dependent transcriptional repressor by preferentially binding to methylated DNA sequences (Nan et al., 1997). As MeCP2 regulates the expression of a multitude of gene products, alteration in MeCP2 activity results in widespread effects (Kriaucionis & Bird, 2003).

MECP2 is carried on the X-chromosome (Amir et al., 1999). Considerable variability in symptom severity occurs in Rett syndrome due to the process of X-inactivation, which results in a mosaic of cells expressing the abnormal gene (Guy et al., 2001). A similar but more severe condition has been described in males as males hemizygous for MECP2 mutation express the abnormal gene in all their cells (Villard et al., 2000).
1.17 MeCP2-null model of Rett Syndrome:

As Rett syndrome appears to be caused by a loss of function of MECP2 protein (Kriaucionis & Bird, 2003) the MeCP2-null mouse has been proposed as a model for Rett syndrome.

Analysis of such a mouse model of Rett syndrome has detected behavioural abnormalities similar to those observed in Rett syndrome sufferers (Guy et al., 2001). Mice developed an abnormal gait, hind limb clasping, exhibited a reduced degree of spontaneous movement and often suffered from breathing abnormalities despite showing no evidence of neurodegeneration (Guy et al., 2001). Despite reports of mild behavioural defects in MeCP2-null mice immediately after birth (Santos et al., 2006), these overt symptoms do not manifest until between 3 and 8 weeks of age (Guy et al., 2001).

1.18 Mitochondrial abnormalities in Rett Syndrome and MeCP2-null mice:

Observations of mitochondrial morphological and biochemical abnormalities in sufferers of Rett Syndrome lead to the suggestion that the disease was a result of mitochondrial dysfunction (Ruch et al., 1989; Eeg-Olofsson et al., 1990; Coker & Melnyk, 1991; Dotti et al., 1993). However attention shifted from this hypothesis following the discovery of high rates of incidence of MECP2 gene mutations in Rett Syndrome sufferers (Amir et al., 1999).

Interest in mitochondrial involvement in Rett syndrome was recently revitalised by the finding that transcription of two components of the mitochondrial electron transport chain was altered in the MeCP2-null mouse model of the condition. In the early symptomatic phase of the disease Uqcrcl (a core subunit of Complex III) was upregulated and later in the disease progression NADH dehydrogenase subunit 2 (a subunit of Complex I) was downregulated (Kriaucionis et al., 2006). The observation of these mitochondrial abnormalities, coupled to the fact that the alterations were only observed in animals that had acquired overt clinical symptoms (Kriaucionis et al., 2006)
indicated that mitochondrial abnormality may play a role in the acquisition of clinical symptoms of Rett Syndrome.

1.19 Aims of the study of Mecp2-null mice:
To examine whether mitochondrial dysfunction participates in the pathogenesis of Rett Syndrome a Mecp2-null mouse model of Rett Syndrome was used. This study sets out to identify whether brain mitochondria from Mecp2-null mice exhibit abnormalities in their morphology or in their respiration rates.
Chapter 2:

*Synaptic efficacy in the PrP-null mouse hippocampus*
Background:

2.1: Studying synaptic efficacy in the PrP-null mouse hippocampus:

The PrP-null mouse provides a model in which to investigate the effects of the loss of the endogenous prion protein, PrPc. Previous studies have described electrophysiological abnormalities in a variety of brain regions in PrP-null mice (Collinge et al., 1994; Colling et al., 1996; Lledo et al., 1996; Carleton et al., 2001; Mallucci et al., 2002; Curtis et al., 2003), although not all reports have been in agreement as to the nature of the phenotype. The hippocampus provides a well characterised brain region which is known to express PrPc at high levels (Sales et al., 1998), and therefore the hippocampal slice provides a useful system in which to study the effects of the loss of PrPc on electrophysiological activity. One electrophysiological parameter that has produced considerable controversy in the literature is the input-output relationship in the Schaffer-Collateral-Commissural Pathway-CA1 synapse in the hippocampus (Lledo et al., 1996; Carleton et al., 2001; Curtis et al., 2003).

An extracellular field potential recording permits assessment of the activity of a large number of neurons surrounding the tip of the microelectrode. To assess the synaptic efficacy of the Schaffer Collateral Commissural-CA1 synapse, the recording electrode is positioned within the Stratum Lacunosum Molecularae of CA1 and the Schaffer Collateral Commissural Pathway is stimulated to produce a field potential recording. Two clearly visible components of such recordings are an initial negative going potential known as the afferent volley, which is followed by the negative going field potential (see Figure 2.5.1). The afferent volley represents depolarization of the axons of Schaffer Collateral Commissural Pathway, and therefore provides a measure of presynaptic activity. When the recording electrode is positioned within the Stratum Lacunosum Molecularae of CA1 the negative going field potential represents dendritic depolarisation of CA1 pyramidal neurons – excitation of the post-synaptic cells. It is therefore possible to assess the relationship between presynaptic activity and postsynaptic activity – the synaptic efficacy.
This Chapter will revisit the question of whether synaptic efficacy is altered in the hippocampus of the Edinburgh PrP-null mouse by studying field potentials in the CA1 region following stimulation of the Schaffer-Collateral Commissural Pathway.
Methods:

2.2: Preparation of hippocampal slices for electrophysiological recording:
Age-matched male PrP-null (Manson et al., 1994) and wild-type control animals were sacrificed by decapitation under halothane anaesthesia. The skull was opened along the sagittal suture line and cuts were made one immediately posterior to the olfactory bulb and another immediately anterior to the cerebellum before the brain was carefully lifted out. The brain was immersed in ice-cold sucrose cutting solution (252 mM sucrose, 2.5 mM KCl, 1.2 mM NaH2PO4, 26 mM NaHCO3, 10 mM MgSO4, 2 mM CaCl2 and 10 mM D-glucose) pre-bubbled with 95% O2/5% CO2 for at least 1 minute. The lateral edges of the brain were trimmed to produce a flat surface parallel to the midline. The tissue was attached to the cutting stage using cyanoacrylate superglue and supported by an agar block. The tissue block was positioned so that the blade would cut through the cortex first with the inferior edge of the brain resting on the agar block. Transverse hippocampal sections 350 μm thick were cut using a 752M vibroslice (Campden Instruments Ltd), whilst the tissue was immersed in cold sucrose slice solution bubbled with 95% O2/5% CO2. Slices were examined under a dissecting microscope and slices without a clear cell body layer were discarded. The cortex surrounding the hippocampus was trimmed from the remaining slices using a razor blade, and the slices were transferred to an interface recording chamber. The aCSF (124 mM NaCl, 2.5 mM KCl, 1.2 mM NaH2PO4, 26 mM NaHCO3, 2.5 mM CaCl2, 1.3 mM MgSO4 and 10 mM D-glucose) in flowing through the recording chamber was continuously bubbled with 95% O2/5% CO2 and was maintained at 30°C ± 0.5. Prior to electrophysiological recording slices were allowed to equilibrate in the bath for a minimum of one hour.

2.3: Electrophysiological Recording:

Electrophysiological recording was carried out using an interface recording chamber. The recording platform was covered with a mesh and overlaid with lens paper to provide support for the tissue. Slices were positioned within the recording chamber (up to six at one time), and the chamber was covered whenever possible to prevent the tissue drying
out. The flow rate of the aCSF from the outlet was adjusted so that the slices sat on the meniscus of the fluid, which equated to an aCSF flow rate of approximately 1-2 ml/minute. Humidified O₂/CO₂ was passed over the slices to maintain them in a moist and oxygenated environment. A temperature of 30°C was maintained within the recording chamber and was continually monitored by a thermal probe. The earth electrode positioned at the interface of the aCSF in the bath provided a reference voltage for electrophysiological recording. The aCSF was collected and recycled to a heated and oxygenated reservoir.

Recording electrodes of 2-10MΩ were prepared from thick walled borosilicate glass capillaries (Harvard Apparatus) using a P87 Flaming Brown Micropipette Puller (Sutter Instruments Co). To record field potentials in the CA1 region of the hippocampus, a stimulating electrode was positioned in the stratum lacunosum-molecularae of the CA3 region, and a recording electrode was positioned in the stratum radiatum of the CA1 region (see Figure 2.3.1). As a general rule, slices were considered viable so long as they demonstrated no epileptiform activity and displayed a clearly visible afferent volley that did not constitute more than one third of the total field potential. The threshold stimulus intensity for the production of a population spike was determined by gradually increasing the stimulus intensity until a population spike was visible as an inflection in the field potential. Similarly the minimal stimulus intensity required for the generation of any detectable EPSP was determined by gradually decreasing the stimulus intensity until no EPSP could be identified. This allowed determination of a suitable increment by which to increase the stimulus intensity so that an input-output curve of at least 6 points could be generated. The stimulus intensity was set to that of threshold for production of an EPSP, and ten consecutive fEPSPs were recorded at 10 second intervals and were averaged. The stimulus intensity was then increased by the required increment and the process repeated until a population spike was clearly visible. All traces containing a spike were disregarded. Data was analyzed online and offline using pClamp 9 software (Axon Instruments Inc.). Linear regressions and 1 way ANOVA
statistical analysis were performed using Sigmaplot 2000 and SigmaStat v2.03 (SPSS inc.) software respectively.

Figure 2.3.1: Main image depicts the principal structures of a transverse hippocampal slice and the positioning of the stimulating and recording electrodes required to detect a field potential to permit construction of an input-output curve for the CA1 region of the hippocampus. The image was acquired using an Olympus BX50WI microscope and Hamamatsu CCD camera whilst the slice was submerged.

2.4: Genotyping of PrP-null mice by PCR and gel electrophoresis:
To validate comparisons between PrP-null and wild-type control OLA129 mice, the genotypes of all mice used for genotype comparison experiments were verified using polymerase chain reaction (PCR) followed by gel electrophoresis.
Tail tips were cut at the time of dissection (using scissors cleaned in ethanol to prevent contamination), and stored at -20°C. DNA was extracted from tail tips using a Genelute mammalian genomic DNA extraction kit (Sigma) following the manufacturers protocol. Briefly two 0.5cm sections of mouse tail were cut and placed in a 1.5ml eppendorf tube. Scissors were cleaned in ethanol prior to cutting tail sections for each animal to minimize contamination. Tail sections were submerged under 200μl lysis solution for tissue (Sigma) supplemented with proteinase K to a final concentration of 2mg/ml, and were incubated at 55°C overnight. Following complete digestion of the tissue, 200μl lysis solution containing chaotropic salts was added to disrupt cells. 200μl 100% ethanol was added and the sample thoroughly vortexed before being transferred to a prepared binding column. DNA was bound to the column by spinning at 6,500g for 1 minute. The column was washed twice using 500μl wash buffer (Sigma), each time followed by centrifugation at 6,500g for 1 minute. After removal of the second batch of wash buffer, the column was dried by centrifugation at 12,000g for 3 minutes. DNA was eluted from the column by addition of 200μl elution solution and centrifuged at 6,500g for 1 minute. Eluted DNA was stored at -20°C.

To differentiate between wild-type and PrP-null OLA129 mice, the neomycin cassette insert (Manson et al., 1994) was amplified by PCR utilizing primers designed to produce a 256 base pair product, which indicates the PrP-null genotype in the Edinburgh Mouse. Primers were: forward 5′ AGC ACG TAC GAT GGA AG 3′ and reverse 5′ AAT ATC ACG GGT AGC CAA CG 3′. A master mixture consisting of 12.5μl Readystart RedTaq DNA polymerase (Sigma), 1.25μl forward primer, 1.25μl reverse primer (each primer to a final concentration of 0.5μM), and 9μl ddH₂O per sample was prepared and supplemented with 1μl of DNA extract (or 1μl ddH₂O for negative control). PCR cycle was 94°C 2min, then 35 repeats of 94°C 30 sec, 58.5°C 30sec, 72°C 20sec.

Amplified DNA was isolated by electrophoresis on a 1.75% agarose (Seakem) in Tris-Borate-EDTA (0.09M Tris HCl pH7.5, 0.09M orthoboric acid, 2mM EDTA pH8.0) gel
containing 1μl ethidium bromide/100ml. A 0 to 2,000 DNA base pair ladder (in increments of 100 base pairs) was run in conjunction with the samples to permit identification of sample fragment length. DNA bands were identified under ultra violet light. Ethidium bromide waste was disposed of as per recommended laboratory procedures.
Results:

2.5: Input-output relationships in the CA1 region of the hippocampus:

Following afferent stimulation extracellular synaptic field potentials were recorded, a typical example of which is shown in Figure 2.5.1. The afferent volley amplitude and the slope of the field potential were measured as shown using pClamp 9 software (Axon Instruments Inc.). With the recording electrode positioned in the stratum radiatum of the CA1 region, negative going field potentials represent dendritic depolarization.

![Figure 2.5.1: A typical recording of an extracellular field potential detected in the CA1 region of the hippocampus.](image)

To investigate the input-output characteristics of the cells in the stratum radiatum, a series of pulses at ascending stimulus intensities were delivered to the CA3 region and the resultant field potential in the CA1 region was recorded. The afferent volley (Figure 2.5.1) is a measure of the depolarisation of the presynaptic axons and hence represents the level of axon recruitment. This was used as the independent variable in the construction of the input-output relationship in order to minimize variability due to positioning of the stimulating electrode. The slope of the field potential (Figure 2.5.1) was used as a measure of the AMPA-mediated post-synaptic response, and is largely unaffected by the generation of any post-synaptic action potentials. The gradient of the input-output relationship constructed by plotting the field potential slope against afferent volley amplitude therefore indicates the magnitude of post-synaptic response to a given pre-synaptic input – the synaptic efficacy of the pathway under investigation.
Figure 2.5.2: Input-Output relationships in the CA1 region of the hippocampus in PrP-null and wild-type mice plotted using the afferent volley amplitude as the independent variable. Panel A - All data points from wild-type (filled circles) and PrP-null (open circles) hippocampal slices. Linear regressions applied to all data within wild-type (blue) and PrP-null (red) data points. Panel B - Linear regressions applied to input-output data from wild-type (blue) and PrP-null (red) hippocampal slices. Gradients of regressions were used for statistical analysis of input-output relationships. Panel C - The mean slopes ± SEM of the input-output relationships constructed for wild-type (black) and PrP-null (grey) slices. Statistical analysis was performed as a 1-way ANOVA with a Tukey Test. ** represents $P < 0.01$. Data was collected from 22 wild-type slices from 6 animals and 25 PrP-null slices from 8 animals.

To analyse the data shown in Figure 2.5.2, a linear regression was applied to the data from each slice, and the gradient of the linear regression determined. Such an analysis technique evenly weights the data from each slice, irrespective of how many data points were acquired to construct the input-output relationship. Following verification that the data passed tests for normality and equal variance, statistical significance was determined by performing a 1-way ANOVA upon the regression slopes for each genotype. A statistically significant difference ($P < 0.01$ 1-way ANOVA) between wild-
type $\left(1.74\text{ms}^{-1} \pm 0.10, n = 22\right)$ and PrP-null $\left(1.33\text{ms}^{-1} \pm 0.08, n = 25\right)$ input-output relationships was detected. This indicates that the postsynaptic response in PrP-null is reduced by approximately 23% relative to control for any given stimulus intensity, and therefore indicates that PrP-null animals possess a lower synaptic efficacy than wild-type controls at the Schaffer Collateral Commissural/CA1 synapse.

As the use of the afferent volley amplitude as the independent variable in the construction of input-output relationships prevents any assessment of axon excitability, the data displayed in Figure 2.5.2 was re-plotted using the stimulation intensity as the independent variable.

**Figure 2.5.3: Input-Output relationships in the CA1 region of the hippocampus in PrP-null and wild-type mice plotted using the stimulus intensity as the independent variable.**

Panel A: All data points from wild-type (filled circles) and PrP-null (open circles) hippocampal slices. Linear regressions applied to all data within wild-type (blue) and PrP-null (red) datasets. Panel B: Slopes of input-output relationships of PrP-null (grey) and wild-type (black) hippocampal slices using stimulation intensity as the independent variable. Error bars represent SEMs. ** represents $P < 0.01$. Data was collected from 21 wild-type slices from 6 animals and 20 PrP-null slices from 7 animals.
Figure 2.5.3 displays input-output data from the CA1 region of the hippocampus of PrP-null and wild-type mice using the magnitude of the stimulus intensity as the independent variable. Data analysis was carried out by determination of the slope of the linear regressions to all the data from a single hippocampal slice, and statistical testing of the slopes using a 1-way ANOVA and Tukey Test. A statistically significant difference ($P < 0.01$) between wild-type and PrP-null input-output relationships was detected. The postsynaptic response in PrP-null slices was reduced by approximately 30% relative to wild-type controls when the input-output relationship was calculated in this way.

2.6: Analysis of field potential half-widths:

To investigate whether the duration of the field potentials was also altered in PrP-null animals relative to wild-type controls, the half-widths of all field potentials were measured. Field potential half-width was found to decrease towards a plateau value with increasing afferent volley amplitude or increasing field potential slope.

Analysis of the relationship between field potential half-width and afferent volley amplitude/field potential slope revealed that the most accurate curve fitting algorithm (highest $r^2$ values) for each data set was an exponential decay 3-parameter fit. The curve fitting algorithm uses the formula:

$$y = y_0 + ae^{-bx}$$

where $y_0$, $a$ and $b$ are constants.

Mathematically the 3 parameters describe three different properties of the curve. The $y_0$ parameter describes the plateau value towards which the curve decreases. The $a$ parameter, in conjunction with $y_0$, describes the $y$-value of the x-intercept. The $b$ parameter describes the rate at which the curve tends towards the plateau value.
Figure 2.6.1: Field potential half widths relative to field slope in the CA1 region of the hippocampus in PrP-null and wild-type mice. Panels A: Relationship between field potential half width and the field potential slope in PrP-null (red) and wild-type (blue) hippocampal slices. Curves were fitted using an exponential decay three parameter curve fitting algorithm. 18 wild-type slices from 6 animals, 18 PrP-null slices from 8 animals. Panel B: Mean parameters ± SEM of curves fitted to half-width versus field slope for each genotype. Statistical testing was performed as a 1-way ANOVA between genotypes. * represents P < 0.05. Panel C: Curves plotted to mean parameters of curves for half-width versus field slope for wild-type (blue) and PrP-null (red) slices.
Exponential decay 3-parameter curves were fitted to the datasets from each individual hippocampal slice. Figure 2.6.1 panel A shows a trend whereby the minimal field potential half-width was reached when the field slope was larger in PrP-null slices when compared to slices from wild-type controls. Statistical testing of the 3 parameters used to fit the curves (Figure 2.6.1 panel B) revealed no significant difference in \( y_0 \) or \( a \), but a statistically significant reduction in \( b \) by almost 50% in PrP-null animals when data was plotted against either field potential slope (Figure 2.6.1 panel B) or afferent volley amplitude (data not shown). These results suggest that whilst the half-width of the field potentials from PrP-null animals appear to decrease towards a similar minimal value \( (y_0) \), they require a significantly larger field potential slope to do so and tend not to have reached this minimal halfwidth of approximately 6ms at the point an action potential is elicited which is indicated by the limit of the plotted input-output relationships and demonstrated by curves fitted to mean parameters shown in Figure 2.6.1 panel C.

When such a curve fitting algorithm is used to analyse the relationship between the field potential halfwidth and the field potential slope, variation in any of these parameters would indicate a discrete event at the level of the synapse. The minimal field potential halfwidth, described by parameter \( y_0 \), occurs when the field potential slope is large (i.e. \( ae^{-bx} \) is small). When the stimulation is such that large field potentials are generated, large numbers of GABAergic feed-forward inhibitory neurons are also activated which act to reduce the field-potential halfwidth. However as the feed-forward inhibitory pathway is disynaptic, whilst the Schaffer-Collateral Commissural-CA1 pathway is monosynaptic, the inhibitory transmission lags behind the excitatory transmission and therefore a minimal field potential halfwidth is reached irrespective of how many feed-forward inhibitory neurons are activated. Alteration of parameter \( y_0 \) may therefore indicate either an alteration in the way in which the cells in this region are connected, or may indicate a different rate of transmission across the synapse. The parameter \( a \), in conjunction with \( y_0 \), describes the y-value of the curve at the x-intercept. Whilst it is not possible to measure this parameter directly, its determination by regression of the curve provides an estimate of what the field potential halfwidth would be in the absence of
GABAergic feed-forward inhibition. Alteration of parameter $a$ would therefore suggest an alteration of the currents responsible for the field potential which could not be attributed to GABAergic feed-forward inhibition. Finally the $b$ parameter describes the rate at which the curve descends towards the plateau $y_0$ value. This therefore describes the level of recruitment of GABAergic feed-forward inhibitory neurons for any given field potential slope.

2.7: Variation in Electrophysiological Data between Experimenters:
Hippocampal slices were prepared by two different individuals for electrophysiological experiments, whilst electrophysiological recording was always performed by the same individual.

Table 2.7.1 compares the data from wild-type and PrP-null hippocampal slices prepared by experimenter 1 and experimenter 2. Input-output relationships in hippocampal slices prepared by experimenter 1 show a robust difference between the genotypes, whilst in those slices prepared by experimenter 2 the difference between the genotypes is considerably smaller. This difference between experimenters appears to be predominantly due to a decrease in the slope of the input-output relationship in wild-type slices.

A similar scenario is observed in the measurements of field potential half-widths, whereby a robust difference between wild-type and PrP-null half-width parameter $b$ was observed in slices prepared by experimenter 1, whilst the difference was considerably smaller in those slices prepared by experimenter 2. Again the difference appeared to be predominantly due to an alteration in the field-potential half-widths in wild-type slices, with those slices of experimenter 2 exhibiting increased field potential halfwidths.
Table 2.7.1: Variation of Input Output relationships and field potential half-widths in PrP-null and wild-type hippocampal slices between experimenters:

<table>
<thead>
<tr>
<th></th>
<th>Experimenter 1 mean ± SEM</th>
<th>n</th>
<th>Experimenter 2 mean ± SEM</th>
<th>n</th>
</tr>
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<tr>
<td><strong>Input-Output Relationship</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>1.96ms⁻¹ ± 0.13</td>
<td>11</td>
<td>1.51ms⁻¹ ± 0.12</td>
<td>11</td>
</tr>
<tr>
<td>PrP-null</td>
<td>1.39ms⁻¹ ± 0.12</td>
<td>13</td>
<td>1.27ms⁻¹ ± 0.09</td>
<td>12</td>
</tr>
<tr>
<td><strong>Field Potential Halfwidth</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parameter b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>9.97 ± 1.67</td>
<td>11</td>
<td>4.18 ± 1.08</td>
<td>11</td>
</tr>
<tr>
<td>PrP-null</td>
<td>4.90 ± 1.08</td>
<td>13</td>
<td>2.90 ± 0.67</td>
<td>12</td>
</tr>
</tbody>
</table>

2.8: Verification of genotypes of experimental animal:

Initial studies demonstrated a bright DNA band of around 250 base pairs in length in PrP-null samples, indicating presence of the neomycin cassette insert. However some dimmer bands (<10% PrP-null band intensity) were observed in wild-type samples, and as negative controls did present the band this indicated either presence of the neomycin cassette insert in wild-type mice, or more likely the contamination of wild-type samples prior to the PCR procedure. Following the implementation of thorough washing procedures for dissection equipment, band intensity in wild-type samples approached that seen in negative controls.

Genotypes could be assigned according to presence or absence of clearly identifiable bands on the electrophoresis gels as shown in Figure 2.8.1. Genotypes were verified for all animals used in genotype comparisons contained within this thesis, and in all cases were as predicted by the breeding program.

It should be noted that primers for a small base pair product (256 base pairs) were utilized within these experiments. This means firstly that fragmentation of the DNA during storage has a lesser affect upon amplification of a small base pair product,
leading to the production of brighter bands on the electrophoresis gel. And secondly the use of primers for a small base pair product in initial experiments allows for the later use of primers for a larger base pair product if a buildup of laboratory contamination with the small product occurs. As primers for a larger product would lie out with the contaminating fragment, amplification of a larger fragment would not be affected by the contamination.

Figure 2.8.1: Example of electrophoresis gel used to determine genotype of wild-type and PrP-null OLA129 mice. Gel contained 1μl ethidium bromide/100ml and was viewed under UV. WT = wild-type, KO = PrP-null, -ve = negative control. Ladder follows 100 base pair increments, bands for multiples of 500 base pairs show greater intensity. PrP-null samples exhibit a bright band between the 200 and 300 base pair bands on the ladder, indicating presence of the neomycin cassette insert. Wild-type samples and negative controls do not show this band.
Discussion:

2.9: Summary of electrophysiological results:

The electrophysiological input-output relationship for the CA1 region of the hippocampus was determined in PrP-null and wild-type mice as a basic measure of synaptic efficacy. The input-output relationship was statistically significantly reduced in PrP-null animals relative to wild-type controls, indicating a reduction in synaptic efficacy. This means that for any given intensity of presynaptic input into the Schaffer-Collateral Commissural Pathway, the magnitude of the post-synaptic response in the CA1 pyramidal cells will be diminished by approximately 25%. In addition the relationship between the field potential slope and the half-width of the field potential was assessed for each brain slice by fitting a three parameter exponential decay curve to each the dataset. Whilst the minimal half-width plateau value was unchanged between the genotypes, the rate of decrease towards this plateau level was significantly attenuated in PrP-null slices relative to wild-type controls. Therefore overall the field potential generated in the pyramidal cells of the CA1 region to any given stimulus intensity will tend to be smaller and will take longer to repolarise back to the resting membrane potential in PrP-null animals.

2.10: Identification of the change responsible for alteration in synaptic efficacy:

A number of factors influence synaptic efficacy and therefore could produce the observed reduction in the input-output relationship in PrP-null mice. Potential presynaptic influences include the excitability of the axons, the probability of synaptic vesicle release and the quantal size of synaptic vesicles released. Postsynaptic factors which could produce the observed difference in the input-output relationship include decreased expression of receptors, decreased conductance of channels or alteration of the $V_m$, thereby creating a reduced driving force through open ion channels. In addition to these presynaptic and postsynaptic mechanisms, an alteration in the number of synapses from each axon could potentially modulate synaptic efficacy.
The use of the stimulus intensity as the independent variable when constructing an input-output curve is subject to considerable variation according to the positioning of the stimulation electrode and the magnitude of the voltage supplied by the batteries within the stimulation pack. In comparison the use of the afferent volley as the independent variable circumvents these problems but does not provide information regarding the excitability of the axons. Both the results presented here and those of Carleton et al (Carleton et al., 2001) detected a significant difference in the input-output relationships between PrP-null and control when the input-output relationship was plotted using either the stimulus intensity or the afferent volley as the independent variable. It can therefore be concluded that an alteration in axon excitability is not responsible for the difference between the genotypes.

Alteration of the probability of neurotransmitter release (pr) is another factor which could influence the synaptic efficacy. As Paired Pulse Facilitation (PPF) correlates inversely with the probability of release in the CA1 region of the hippocampus (Wasling et al., 2004), along with the fact that differences in PPF in the CA1 region of the hippocampus have not been observed in either the Zurich (Carleton et al., 2001) or the Edinburgh (Curtis et al., 2003) PrP-null mouse, alteration in the probability of neurotransmitter release can be discarded as a potential mechanism responsible for the phenotypic difference.

The amount of neurotransmitter released in a single vesicle has been demonstrated to be modulated (Burgoyne & Barclay, 2002). A reduction in the quantal size of neurotransmitter released in PrP-null animals could potentially explain the observed difference in the input-output relationships by reducing the postsynaptic response to the release of a given number of vesicles. However no evidence to date has been published to support this possibility in PrP-null mice.

In order for a postsynaptic mechanism to cause reduction of the electrophysiological input-output relationship in PrP-null mice, the ionic flux across the plasma membrane
would have to be decreased. This may occur via either decreased expression of receptors or a decreased flux through receptor channels either due to a decreased driving force, or decreased channel conductance. The membrane potential ($V_m$) contributes towards the driving force for ion movement. CA1 pyramidal cells of the Zurich PrP-null mouse have been shown to possess an unaltered membrane potential (Colling et al., 1996) and following postnatal knockout of the prion protein the membrane potential of the CA1 pyramidal cells has been shown to be unchanged (Mallucci et al., 2002). This suggests that an alteration in driving force is unlikely to be responsible for the observed difference in the input-output relationship. As the slope of the field potential was measured (rather than the field potential amplitude), our result reflects a difference in the strength of the AMPA response to a given stimulus (Johnston & Miao-Sin Wu, 1995). Whilst a decrease in AMPA receptor expression provides a reasonable explanation for the decreased input-output relationship in PrP-null mice, to date no published studies have reported alterations in AMPA receptor subunit expression in PrP-null animals. However a recent study reported a 1.2 fold decrease in the expression of the AMPA3 receptor subunit in scrapie infected mice. This observation was consistent across mice infected with three different strains of scrapie, and was not detected in scrapie inoculated mice prior to the acquisition of clinical symptoms (Skinner et al., 2006). This reduced expression of an AMPA receptor subunit, in conjunction with the relationship between scrapie infected and PrP-null mice, suggests that reduced AMPA receptor expression may underlie the observed reduction in synaptic efficacy in PrP-null mice.

Alterations of NMDA receptor subunit expression have been detected in the dentate gyrus of the hippocampus of Zurich PrP-null mice (Maglio et al., 2004). However this phenomenon cannot explain the observed reduction of the input-output relationship in PrP-null animals, as an increased NMDA channel current would be expected to enlarge the field potential for any given afferent stimulation, and because NMDA currents are excluded from measurements of field potential slope.
Decreased inhibitory post-synaptic currents have been observed in the cerebellum of Zurich PrP-null mice following the application of copper ions (Brown et al., 1997a). This may reflect the loss of a copper buffering capacity resulting in increased copper concentrations which modulate synaptic activity. The differences in input-output relationships observed may therefore be due to the modulation of synaptic transmission by copper ions. However the experiments of Brown et al (Brown et al., 1997a) only detected a significant difference between PrP-null and wild-type controls upon the application of exogenous copper ions, suggesting that a phenotype would not be observed prior to copper addition. As this effect was observed following addition of CuSO₄ to a concentration of 2μM, such an effect is unlikely to have occurred due to copper contamination of buffer solutions.

A reduction in the number of synapses of each of the Schaffer Collateral Commissural Fibres on the CA1 pyramidal cells could also provide an explanation for the observed reduction in Synaptic Efficacy. A role for PrP<sup>C</sup> in synaptogenesis has been proposed due to the spatial and temporal correlation between PrP<sup>C</sup> expression and axon development/synapse formation (Sales et al., 2002). The loss of PrP<sup>C</sup> in the PrP-null animals could therefore result in reduced synapse formation and thereby produce the observed synaptic efficacy deficit. Such losses of synapses, axons and dendritic spines have been observed in a number of models of scrapie infection (Jeffrey et al., 1995; Jeffrey et al., 2000; Belichenko et al., 2000; Brown et al., 2001; Bouzamondo-Bemstein et al., 2004) suggesting that synapse loss may be a cause of abnormalities in synaptic transmission in scrapie. However a published study of PrP-null mice has reported increased sprouting of fibres and a reduction in neuronal excitability in the dentate (Colling et al., 1997), thereby suggesting that increased a reduction in synapse formation is unlikely to underlie the electrophysiological abnormalities reported in this study.

Evidence that PrP<sup>C</sup> is expressed presynaptically (Sales et al., 1998; Herms et al., 1999) initially suggests that the observed alteration would be due to a presynaptic effect.
However it remains possible that the loss of PrP \( ^{C} \) indirectly causes the alteration in synaptic efficacy and therefore the possibility that the observed difference is due to a postsynaptic effect cannot be excluded. It therefore appears that the reduction in synaptic efficacy in the PrP-null mouse is not due to altered axon excitability, probability of neurotransmitter release or \( V_m \), although the exact mechanism, whether presynaptic, postsynaptic or altered number of synapses, remains elusive.

2.11: Alterations in field potential half-width:

Field potential halfwidths were observed to decrease towards a plateau value with increasing field potential size. This relationship between field potential slope and field halfwidth is in agreement with the findings of Turner, which suggest that increasing recruitment of GABAergic feed-forward inhibitory neurons by the Schaeffer Collateral Commissural Fibres results in a truncation of the field EPSP (Turner, 1990). The precise nature of the change observed between the genotypes indicates that whilst the minimal field potential half-width is unchanged, the field potential half-width in PrP-nulls declines more slowly towards this plateau value with increasing field potential size when compared to the wild-type controls.

Modeling of the field EPSP in the CA1 region of the hippocampus has suggested that the decaying phase (and therefore halfwidth) of the field EPSP is determined by GABA and NMDA currents (Sargsyan et al., 2001). In order to increase the halfwidth of the field EPSP an increase in NMDA receptor currents or a reduction in GABA receptor currents would be necessary. Other labs have published results which lend support to each of these potential mechanisms. Collinge et al identified a reduction in GABA_A receptor mediated fast-inhibition in the hippocampus of the Zurich PrP-null mice (Collinge et al., 1994), although similar experiments in the cerebellum have found no alteration in GABA_A mediated currents (Herms et al., 1995). Maglio et al have identified increased expression of NMDA receptor subunits NR2A and NR2B in the hippocampus of the Zurich PrP-null mouse (Maglio et al., 2006), although they did not test whether their upregulation lead to increased ionic fluxes through NMDA channels. However it is
likely that the NMDA receptors will be subject to a voltage-dependent magnesium blockade under our experimental conditions and so an increased ionic flux through NMDA receptors is unlikely to cause the increase in field potential halfwidth. Therefore the likely explanation for the increased field potential halfwidths in PrP-null mouse hippocampus is a loss of GABA_A-mediated hyperpolarisation.

A reduction in synapse formation provides a mechanism by which the field potential half-width could have increased in PrP-null hippocampus. PrP_C has been associated with synaptogenesis and a loss of synapses is observed in scrapie infected mice (Brown et al., 2001). A reduction of the number of synapses from either the Schaffer Collateral Commissural Fibres onto the GABAergic interneurons, or from the GABAergic interneurons onto the CA1 pyramidal cells would result in a reduction in feed-forward inhibition and thereby widen the field potential. This mechanism is supported by evidence indicating a loss of GABAergic synapses following scrapie inoculation (Bouzamondo-Bernstein et al., 2004). Alternatively a decrease in the expression of AMPA receptors, as suggested to occur following scrapie inoculation (Skinner et al., 2006), may result in reduced activation of GABAergic feed-forward inhibitory neurons and thereby broaden the field potential.

Alternatively a reduction in GABAergic feed-forward inhibition of the CA1 pyramidal cells may be due to decreased expression of GABA_A receptors. A study of gene expression revealed a 1.2-fold decrease in the expression of the GABA_A receptor subunit g2 which was consistent across groups of mice inoculated with 3 different strains of scrapie (Skinner et al., 2006). If downregulation of this subunit results in decreased GABA_A feed-forward inhibitory currents, the widening of the field potential observed in PrP-null mice may be due to this reduction in GABA receptor subunit expression.
2.12: Experimenter to experimenter variability in detection of electrophysiological abnormalities in PrP-null mice:

Data presented in this thesis strongly suggests experimenter to experimenter variability in the detection of electrophysiological abnormalities in PrP-null mice. These differences centered upon altered synaptic efficacy and field potential halfwidth in wild-type slices, whilst PrP-null slices remained relatively unchanged between experimenters. Previous studies have indicated that GABAergic interneurons are more susceptible to damage during hippocampal slice preparation (Kuenzi et al., 2000). The detection of a significant difference between the genotypes may therefore rely upon the preservation of GABAergic interneurons during slicing. An increased susceptibility of GABAergic neurons to damage during slicing may also help account for the earlier contradictory reports of electrophysiological abnormalities in PrP-null mice such as the detection/failure to detect a difference in synaptic efficacy (Lledo et al., 1996; Carleton et al., 2001).

2.13: Other reports of input-output relationships in PrP-null mice:

The ability to detect differences in the input-output relationship for the CA1 region of the hippocampus of PrP-null mice appears to be variable between experimenters. It has previously been reported that the Zurich strain of PrP-null mouse exhibited normal neuronal excitability (Lledo et al., 1996), although the same group later demonstrated a facilitation of synaptic transmission proportional to the level of expression PrP<sup>C</sup> expression (Carleton et al., 2001). This finding resembles our results whereby no difference in the input-output relationship was initially detected in the CA1 region of the hippocampus of the Edinburgh PrP-null mouse (Curtis et al., 2003), whilst later experiments reported here demonstrated a clear statistically significant reduction in synaptic efficacy in PrP-null mice. As all experiments from our laboratory were performed under the same conditions, the differences in the results reported may be due to variability in hippocampal slice preparation.
Carleton et al. have also reported that the difference in the input-output relationship between PrP-null and wild-type animals is not evident by the time that the animals are 10-14 months old (Carleton et al., 2001). Whilst the current study did not examine the input-output relationships of aged PrP-null mice, previous reported results from our laboratory have indicated reductions in both Post-Tetanic Potentiation (PTP) and Long-Term Potentiation (LTP) in the CA1 region of the hippocampus which were evident in animals aged approximately 10 months, but not in young adults (Curtis et al., 2003). Despite the striking coincidence that one alteration in synaptic function is lost whilst another is gained, the issue of whether these phenomena are linked remains unresolved.

2.14: Implications of the Prion protein forming ion channels:
Evidence for the prion protein fragment [106-126] forming channels in lipid bilayers was first reported in 1997 (Lin et al., 1997). These channels have been characterised as passing potassium and chloride currents (Kourie & Culverson, 2000), and thereby their activity results in hyperpolarisation of the cell. The lack of expression of prion protein in the PrP-null mice would presumably result in the loss of any such PrP channels. Various effects of the loss of such channels can be envisaged. The loss of a channel which hyperpolarises the cell could be predicted to facilitate synaptic transmission, which is in contrast to the observed reduction in input-output relationship observed in PrP-null animals. Whilst the loss of a prion protein channel initially may appear to provide a possible explanation for the increased half-width of field potentials in PrP-null animals, the presynaptic localization of PrP (Herms et al., 1999) means that such channels would be unable to modulate the halfwidth of the field EPSP. However alternative schemes can be envisaged whereby the loss of PrPC channels could produce the observed reduction in synaptic efficacy. These include the possibility that chronic depolarisation of the synaptic boutons due to the loss of a PrPC channel could result in a decreased driving force for calcium entry and therefore reduce synaptic efficacy, and the loss of the normal PrPC expression in axons (Barmada et al., 2004) resulting in an absence of PrPC channels could in chronic depolarisation of the axon and blocking of action potential propagation. However all of these hypotheses have to be treated with
caution as it is difficult to interpret the action(s) of any PrP$^C$ ion channels in vivo from studies of channels in lipid bilayers.

2.15: Electrophysiological abnormalities in TSE's:
Electrophysiological abnormalities seen in TSE disease states could occur in the PrP-null mouse model if TSE pathogenesis is at least in part dependent upon the loss of PrP$^C$ function. Electrophysiological studies of mice infected with the ME7 scrapie agents have revealed that scrapie infected mice possess a clear electrophysiological phenotype which develops over time following inoculation with the ME7 scrapie agent (Johnston et al., 1997; Johnston et al., 1998; Chiti et al., 2006). The stimulus intensity required to produce a 50% fEPSP was increased (Johnston et al., 1997; Chiti et al., 2006), and the size of the field potential decreased (Johnston et al., 1998) in scrapie infected mice, indicating a decrease in neuronal excitability as seen in PrP-null mice. However in contrast to the observations in PrP-null mice, no difference in the input-output relationship in the CA1 region of the hippocampus was detected when field slope was plotted against afferent volley (Chiti et al., 2006), although the field potentials analysed in this study were exceedingly small. Both the fast and slow I$\text{AHP}$ has been observed to be increased following scrapie infection (Johnston et al., 1997; Chiti et al., 2006), a finding which is in contrast to the decreased I$\text{AHP}$ observed in PrP-null mice (Colling et al., 1996). Alterations in synaptic plasticity have also been observed with scrapie infected mice exhibiting attenuated LTP (Johnston et al., 1998; Chiti et al., 2006) and a trend towards decreased PTP (Chiti et al., 2006). Therefore overall the electrophysiological abnormalities observed in PrP-null mice are similar, with a few notable striking differences, to those observed following scrapie infection. This suggests that electrophysiological abnormality in TSE's could at least in part be caused by the loss of PrP$^C$. 
Chapter 3:

Neuronal Survivability in the PrP-null Hippocampus
Background:

3.1: Studying neuronal survivability in organotypic cultures of PrP-null mouse brain:
Previous studies have suggested that neurons of PrP-null mouse brain are more prone to cell death in cell culture systems (Brown et al., 1997b; Brown et al., 1998; Brown et al., 2002; Kim et al., 2004). The slow-onset of many neurodegenerative diseases thought to involve oxidative stress suggests the chronic accumulation of oxidative damage, rather than an acute oxidative insult, underlies these conditions (reviewed in (Halliwell, 2006). It is therefore useful to be able to study neuronal survivability under cell culture conditions which permits experiments to be conducted over a longer time period than would be possible in acute slice preparations. Previous studies of neuronal survivability in the absence of PrP have used dissociated cell cultures or cell lines as their experimental system. This has not permitted conclusions to be reached as to whether subregions of the hippocampus are more or less susceptible to degeneration in cell culture.

The organotypic cell culture maintains a degree of structure, thereby permitting conclusions to be reached regarding the regional vulnerability of hippocampal subregions to cell death under culture conditions. In addition the retention of synaptic connections in organotypic cultures permits electrophysiological studies to be performed upon the cultures (although this is out-with the scope of this thesis).

The physical nature of organotypic cell cultures permits assessment of cell death rates using a variety of different assays, such as propidium iodide staining, Fluoro-Jade staining or lactate dehydrogenase efflux (Noraberg et al., 1999). This study will employ the propidium iodide staining technique as a general marker of cell death to assess levels in different subregions of the hippocampus of organotypic cell cultures of PrP-null mice.

It has previously been reported that cell death rates of PrP-null neurons are particularly high under pro-oxidative conditions (Brown et al., 1998; Kim et al., 2004). In order to control the composition of the culture medium, a defined Neurobasal + B27 supplement
medium (Invitrogen) will be used within these experiments. This provides the opportunity to selectively withdraw antioxidants from the culture medium due to the availability of different B27 supplements, and therefore determine whether PrP-null neurons are indeed intrinsically susceptible to oxidative insults.

This Chapter will therefore address whether the survivability of neurons in the PrP-null hippocampus is altered, whether pro-oxidative conditions are required for a phenotype to be detectable, and whether the neurons of the hippocampus display regional variability in their susceptibility to degeneration.
Methods:

3.2: Preparation of Organotypic Slice Cultures:
Organotypic hippocampal slice cultures were prepared by Dr John Curtis. The organotypic cell culture technique used to culture hippocampal slices was broadly based upon the static culture method of Stoppini et al (Stoppini et al., 1991). Measurement of levels of neuronal death following experimental manipulation using propidium iodide was based upon the work of Noraberg et al (Noraberg et al., 1999).

Organotypic hippocampal slice cultures were prepared from 7-8 day old PrP-null and wild-type control mice. Animals were sacrificed by decapitation under halothane anaesthesia. Brain tissue was kept cold during dissection using ice cold cutting solution (252mM sucrose, 2.5mM KCl, 1.2mM NaH2PO4, 26mM NaHCO3, 10mM D-glucose, 2mM CaCl2, 10mM MgSO4). Hippocampi were dissected out taking care to dissect away the meninges and to avoid causing mechanical damage to the tissue. Dissected hippocampi were placed into 4°C Hibernate A solution (Brain Bits Ltd.) whilst the dissection was completed. To ensure sterility all subsequent procedures were carried out in tissue culture hoods.

Hippocampi were positioned on a double layer of filter paper (Whatman no. 1) soaked in Hibernate A on the stage of a McIlwain Tissue Chopper. Transverse hippocampal slices of 350μm thickness were cut using the tissue chopper, and carefully separated using a brush and smoothed spatula. Following separation, slices were placed into a Hibernate A filled Petri dish and left in the fridge for 1 hour.

1ml Opti-MEM medium (50% Opti-MEM, 25% HBSS, 25% horse serum, 125U/ml penicillin-streptomycin, 25mM glucose, sterile filtered, all supplied by Invitrogen with the exception of glucose supplied by BDH) was added to the appropriate number of wells of 6-well plates, and 30mm 0.4μm pore size organotypic cell culture inserts (Millipore) were placed in the wells to wet for a minimum of 30 minutes prior to the application of slices. Following 1 hour under Hibernate A to allow toxins to diffuse out
of the tissue, slices were transferred to Organotypic cell culture inserts, up to 5 slices per insert, using a fire-polished glass dropper. Excess fluid was carefully removed from around each slice using sterile fine-tipped pastettes. These procedures were carried out as rapidly as possible to prevent the slices drying out.

3.3: Maintenance and experimental manipulation of Organotypic Slice Cultures:
Organotypic cultures were kept in a tissue culture incubator set at 33°C and 5% CO₂. Medium was completely exchanged every 2-3 days to prevent build-up of metabolites. Following 5 days in culture, the serum based optimem medium was exchanged for a defined Neurobasal A + B27 supplement medium (98ml Neurobasal A Medium, 2ml B27 supplement, 1mM L-glutamine, penicillin streptomycin, 25mM D-glucose all supplied by Invitrogen except glucose supplied by BDH). For the initial 10 days in serum-free medium, all cultures were maintained in a medium which included B27 supplement containing the antioxidants superoxide dismutase, catalase, reduced glutathione and α-tocopherol (Brewer et al., 1993). Following a period of 10 days in serum-free medium, cultures were placed either in Neurobasal A medium supplemented with B27 with or without antioxidants. Imaging of cultures commenced 3 days after exposure to plus or minus antioxidant medium.

To determine cell death in organotypic slice cultures, the dye propidium iodide was added to a final concentration of 2μM in the culture medium for 3 hours prior to image acquisition. Organotypic hippocampal slice cultures may contain different densities (and therefore numbers) of neurons. To permit normalization to the number of neurons present, initial images were normalized by expressing them as a percentage of total possible neuronal death. This was achieved using the neurotoxic properties of glutamate. Following acquisition of initial images, glutamate to a final concentration of 50mM was added to the medium. Following 1 hour of exposure to glutamate, the medium was changed to the appropriate Neurobasal + B27 medium (plus or minus antioxidants) including PI to a final concentration of 2μM to remove glutamate.
24 hours after the addition of glutamate, the organotypic slice cultures and the beads were re-imaged. The time-course of organotypic cell culture experiments is summarised in Figure 3.3.1.

Figure 3.3.1: Timeline of organotypic cell culture experiments. Note that routine changes of medium to maintain cultures are not shown.

3.4: Imaging of Organotypic Slice Cultures:
Organotypic slice cultures were viewed through an Olympus BX50WI microscope using a 4x magnification objective lens. For fluorescence imaging, slices were excited at 545nm using a Photonics LPS150 monochromator and imaged using a Hamamatsu ORCA-ER CCD camera. Images were acquired using Simple PCI software (Compix Inc.). Standard PCI software settings for image capture were contrast offset -22, receiver gain 10, exposure time 0.9576 seconds. Due to the importance of keeping all images in focus, focusing was carried out using the microscope controller interface in the imaging software. Images of subregions of each hippocampal slice (CA1, CA3 and dentate gyrus) were acquired in order to centre each image and thereby prevent any potential variation due to an uneven light source and to ensure that all of the subregion was captured within the field of view.

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In order to normalize images to any alterations in the strength of the excitation light source, fluorescent beads (30% intensity Inspeck Red 580/605 2.5µm diameter, Molecular Probes) were added to each culture insert and imaged both before and after imaging of organotypic slice cultures. Fluorescence intensity of beads was determined using ImageJ software as the maximal greyscale pixel intensity of objects identified as single beads. As a wavelength of 545nm was to be used to excite PI in hippocampal slices, this same wavelength was used to excite the fluorescent beads despite it producing suboptimal excitation. The use of this wavelength is beneficial as any variation in the intensity of different wavelengths emitted by the light source will not affect the normalization procedure.

The exposure time of 0.9576 seconds was selected as this produced non-saturated images of PI fluorescence following glutamate treatment of organotypic slice cultures. However the use of this exposure time produced low intensity images which could not be aligned accurately by eye. To permit alignment of images for pre and post-glutamate treatment, a transmitted light image was taken in an identical position to each fluorescent image. Transmission images were then aligned to each other, and alignment of each fluorescent image to its transmission partner and locking of the images in a stack permitted alignment of low intensity fluorescent images using Photoshop 7.0 (Adobe).

Mean pixel intensity analysis of areas of interest was carried out using ImageJ software (NIH). Regions of interest were selected in the post-glutamate treatment fluorescent images, and copied onto pre-glutamate treated images to ensure identical regions of each culture were assessed before and after glutamate treatment, and PI fluorescence within these selected regions was measured. As the image background intensity was not zero, the mean pixel intensity of a region of each image which showed no PI fluorescence was subtracted from the mean pixel intensities of the regions of interest. Cell death prior to glutamate treatment was calculated as the level of fluorescence prior to glutamate treatment divided by the level of fluorescence following glutamate treatment following normalization of fluorescence levels and subtraction of background fluorescence.
Results:
3.5: Normalization of propidium iodide uptake images using fluorescent beads:
In order to permit normalization of fluorescence across the experimental time course using Inspeck red fluorescent beads, it was necessary to ensure that there was a linear correlation between bead fluorescence intensity and fluorescence intensity from propidium iodide treated hippocampal slices whilst varying the sensitivity of the imaging equipment. To this end, a sample of 30% intensity fluorescent beads and a glutamate plus PI treated hippocampal slice were imaged using a variety of exposure times.

Figure 3.5.1: Normalization of fluorescence intensities using fluorescent beads. Panel A: Image of fluorescence beads using standard image acquisition settings. Panel B: Magnified image of fluorescent beads used to identify single beads for fluorescence intensity analysis. Panel C: Comparison of fluorescence intensities from PI fluorescence of the Dentate Gyrus (filled circles) and CA1 region (open circles) of a hippocampal slice against fluorescent beads. Multiple exposure times were used for image acquisition to permit multiple images to be taken from the same slice. Fluorescence intensity was analysed for 20 beads for each point, error bars represent SEM. The images from hippocampal slices of points labelled * were saturated.
The data in Figure 3.5.1 panel C demonstrates the relationship between fluorescence intensity from fluorescent beads and PI in a hippocampal slice. At long exposure times the images from hippocampal slices were saturated and were discounted from further analysis.

Following discounting of all saturated images, Figure 3.5.1 panel C demonstrates a linear relationship between the fluorescence intensities measured from the fluorescent beads against PI in hippocampal slices ($r^2 > 0.99$ for both subregions analysed). As a linear relationship exists between the measured fluorescence intensities from the beads and the slice, offline normalization to bead intensity is validated as a suitable means to account for day to day fluctuations in the fluorescence setup. To ensure that bead fluorescence intensity was not altered during the 24 hour period they were in the culture plate or by incubation in different media, bead fluorescence intensities for all experimental combinations were compared.

The data in Figure 3.5.2 demonstrates that bead fluorescence intensity did not alter significantly either with time in culture, nor with exposure to plus or minus antioxidant B27 medium and further validates the bead fluorescence intensity measures as a normalization tool.

The lack of difference in bead fluorescence intensity between inserts loaded with wild-type and PrP-null slices is reassuring as it indicates that normalization to bead intensities is unlikely to produce an artefactual difference between the genotypes.
Figure 3.5.2: Comparison of bead fluorescence intensities (average of 20 from each insert) under different conditions. Panel A: Comparison of bead fluorescence intensities on the first days imaging experiments (dark-grey, n =12) with bead fluorescence intensities from day 2 imaging experiments following glutamate exposure (light-grey, n =12). Panel B: Comparison of bead fluorescence intensities from inserts incubated in Neurobasal A medium supplemented with B27 with antioxidants (dark-grey, n =14) and B27 minus antioxidants (light-grey, n =10). Panel C: Comparison of bead fluorescence intensities from inserts containing wild-type (dark-grey, n =12) and PrP-null (light-grey, n =12) slices. Statistical testing using 1-way ANOVA failed to detect any significant differences between bead fluorescence intensities under any of the conditions tested.
3.6: Assessment of cell death by fluorescence imaging of PI uptake in defined regions of the hippocampus in PrP-null and wild-type control mice:

As it was possible to normalize fluorescence intensities between days reliably, an assessment of neuronal death in cell culture was carried out by determining the percentage death of the total neuronal population within each culture.

Data is presented in Figure 3.6.1 for the percentage cell death during cell culture for PrP-null and wild-type organotypic hippocampal slices incubated in medium containing plus antioxidant or antioxidant-free B27 supplement. As the CA3 region of the hippocampus displayed poor levels of structure following glutamate treatment, it proved impossible to achieve an accurate assessment of cell death in of the CA3 region of organotypic cell cultures. The CA1 and dentate gyrus regions routinely displayed clear structure permitting analysis of cell death in culture. Slices presenting no clear structure following glutamate treatment were discounted from further analysis.

Analysis of PrP-null and wild-type slices cultured in Neurobasal A medium with B27 plus antioxidants showed no statistically significant difference in percentage cell death rates between genotypes in either the CA1 region or the dentate gyrus. However when slices were cultured in the same medium lacking antioxidants a statistically significant increase in cell death was observed in the CA1 region of the hippocampus, but not in the dentate gyrus, in PrP-null mice. This indicates a selective sensitivity of the CA1 region to oxidative stress in PrP-null mice. Whilst statistically the difference between genotypes appears very robust, caution must be applied when interpreting these results due the fact that multiple slices were obtained from multiple animals within each experiment thereby reducing the effective n number. For experiments on wild-type and PrP-null slices in plus antioxidant medium and wild-type slices in minus antioxidant medium, slices were prepared from 3 animals across two culturing procedures, and in the case of PrP-null slices in minus antioxidant medium, slices were prepared from 2 animals in a single culturing procedure. Therefore the number of culturing experiments and number of animals used was considerably lower than the number of slices assessed.
Figure 3.6.1: Comparisons of magnitudes of cell death in hippocampal organotypic cell cultures of PrP-null and wild-type mice. Panel A: Aligned images of the CA1 region of a wild-type organotypic cell culture before (top) and after (bottom) glutamate treatment. Panel B: Comparison of percentage cell death rates in organotypic culture for the CA1 region of the hippocampus from PrP-null (grey) and wild-type (black) mice in medium supplemented with B27 with or without antioxidants. \( n = 13 \) wild-type with antioxidants, \( n = 19 \) PrP-null with antioxidants, \( n = 11 \) wild-type without antioxidants, \( n = 10 \) PrP-null without antioxidants. Panel C: Aligned images of the dentate gyrus of a wild-type organotypic cell culture before (top) and after (bottom) glutamate treatment. Panel D: Comparison of percentage cell death rate in organotypic culture for the dentate gyrus of the hippocampus from PrP-null (grey) and wild-type (black) mice in medium supplemented with B27 with or without antioxidants. \( n = 13 \) wild-type with antioxidants, \( n = 19 \) PrP-null with antioxidants, \( n = 12 \) wild-type without antioxidants, \( n = 10 \) PrP-null without antioxidants. Statistics were performed as 2-way ANOVA with Tukey test across genotype and medium type. *** represents \( P < 0.001 \).
As the mean pixel intensity assessment of fluorescence intensity can be affected by the size of the area selected, an assessment of the sizes of the regions of interest selected for wild-type and PrP-null mice was carried out. Whilst it is possible that a statistically significant difference may result from an alteration of brain size, or altered spread of organotypic cultures, an alteration in the sizes of the regions assessed could indicate a systematic error leading to the production of a false positive result.

Figure 3.6.2: Comparison of the areas of the regions of interest for PrP-null (grey) and wild-type (black) CA1 region and dentate gyrus of organotypic cell cultures. Data is presented relative to the area of the wild-type for the appropriate region of interest. Statistical testing using 1-way ANOVA failed to detect a significant difference between the areas selected for wild-type and PrP-null cell cultures.

The data in Figure 3.6.2 demonstrates that the regions of interest selected for each hippocampal subregion showed no significant difference between wild-type and PrP-null mice. This indicates that no systematic over or underestimation during the selection of regions of interest is likely to be responsible for the observed difference between genotypes.

Furthermore all images were contaminated with a level of background fluorescence. If this is not accounted for during analysis, the percentage cell death prior to glutamate
addition will be overestimated. In order to subtract background fluorescence from measured pixel intensities from brain subregions, a region of interest was positioned at points surrounding the hippocampal subregion taking care not to include any spots of PI fluorescence. As this background subtraction procedure could have considerable impact upon the results, an assessment of the variability of this background measure was carried out in five slices. It was determined that whilst the background levels of fluorescence varied to some degree between slices, the variation of intra-slice repeated background measures was considerably smaller (data not shown). The maximal variation in background intensity observed within a set of five measures equates to approximately 10% of the mean change in pixel intensity following glutamate treatment. The implication of this finding is that background fluorescence adds an extra source of noise to the assessment of cell death by PI uptake, but the added noise is only a minor contributor towards variability between experiments. Additionally mean background intensity was not found to vary over the duration of the experiments, whether the medium contained antioxidants, or with the genotype of the slices (data not shown). This indicates that variability in background levels of fluorescence are unlikely to contribute to the observed difference between genotypes.
Discussion:

3.7: Summary of results of organotypic cell culture of hippocampal slices from PrP-null mice:

To assess whether PrP-null animals were more susceptible to oxidative stress-induced neuronal death, PrP-null and wild-type mouse organotypic hippocampal cultures were assessed using PI uptake to indicate neuronal death. No difference in PI uptake was observed between the genotypes when organotypic cultures were maintained in an antioxidant supplemented medium. However, when organotypic cultures were maintained in a culture medium lacking antioxidant supplementation, an increased rate of cell death was observed in the CA1 region, but not the dentate gyrus, of PrP-null mice. Whilst the n numbers for these experiments are low when considering the number of culture procedures performed, the lack of change in cell death rates in the dentate gyrus of PrP-null slices acts as an internal control thereby strengthening the difference between cell death rates in the CA1 region.

3.8: Does increased PI uptake represent increased neuronal death?

Propidium iodide is a polar compound which only enters cells following permeabilisation of the plasma membrane. Once PI gains access to a cell it can bind to DNA where it fluoresces brightly. Therefore PI fluorescence indicates permeabilization of the plasma membrane and is considered a marker of cell death. This mechanism of action does not make PI staining specific for degenerating neurons, but rather it provides an assessment of the number of damaged neurons and glia. This means that in the current study the initial measure of PI uptake represents total cell death rather than solely neuronal death. It is therefore impossible to determine whether the increased level of PI uptake in PrP-null organotypic cell cultures is due to an increase in neuronal or glial cell death. However, all data was normalized to total neuronal number present via addition of a neurotoxic dose of glutamate. Therefore, if a significantly increased proportion of glia were dead prior to glutamate addition it would be expected that raw post-glutamate fluorescence intensities would be increased in PrP-null cultures due to the additive effect of pre-glutamate treatment glial death and post-glutamate treatment...
neuronal death. As no significant difference in PI fluorescence intensities was observed between PrP-null and wild-type controls following the addition of glutamate, and because the regions of interest selected contain a high proportion of neurons, it is unlikely that the increased PI fluorescence intensities observed in PrP-null organotypic cell cultures were due to an increase in glial death.

Whilst the addition of glutamate was assumed to result in death of all neurons in organotypic cell cultures as suggested by the methods of Noraberg et al (Noraberg et al., 1999) and verified by our pilot experiments (data not shown), it is possible that the observed difference between genotypes could be caused by PrP-null cell cultures exhibiting a decreased sensitivity to glutamate. However this would require a reduction in levels of PI uptake (indicating cell death) following glutamate treatment of PrP-null cultures to produce the observed result. As PI fluorescence following glutamate addition was unchanged in PrP-null cultures relative to wild-type controls, and because previous studies have indicated that cerebellar PrP-null cells exhibit increased vulnerability to glutamate toxicity (Brown et al., 2002), a reduction in the glutamate sensitivity of PrP-null organotypic cultures is unlikely to have produced the observed difference.

3.9: Susceptibility of PrP-null hippocampal organotypic cell cultures to oxidative stress: The data presented in this thesis demonstrates increased cell death in PrP-null hippocampal cultures when subjected to pro-oxidant conditions via antioxidant withdrawal. This is in agreement with previous reports that PrP-null mice show an increased susceptibility to oxidative stress, including that cultures of cerebellar cells from PrP-null animals exhibit a reduced survivability in pro-oxidative conditions (Brown et al., 1997b; Brown et al., 2002), that cerebellar cell cultures of PrP-null animals show increased susceptibility to copper toxicity (Brown et al., 1998) and that cerebellar cultures show an increased susceptibility to hydrogen peroxide toxicity (White et al., 1999).
Another result supporting the potential involvement of oxidative stress in increased cell death rates in PrP-null cell cultures is that a hippocampal PrP-null neuronal cell line exhibited greater rates of apoptosis upon the withdrawal of serum from the culture medium (Kim et al., 2004). Whilst serum contains a variety of compounds which may aid in cell survival, a number of constituents are antioxidants. As the withdrawal of serum resulted in increased rates of cell death, it is possible that the reduction in antioxidant protection is responsible for the increased rates of cell death in the PrP-null cultures and thereby mirrors the results presented in this thesis. However the use of serum supplementation of the medium, rather than the use of a defined medium as in this study, means that it is not possible to definitely implicate antioxidant withdrawal as the cause of increased rates of cell death in PrP-null cultures as observed by Kim et al. The study of Kim et al also highlighted the importance of mitochondrial depolarization and calcium accumulation in the apoptotic cascade in PrP-null cultures (Kim et al., 2004) thereby suggesting a role for mitochondria in the observed cell death, although whether mitochondrial abnormality is the cause of the apoptotic cell death observed is unresolved.

In the study reported here cultures were maintained in serum containing medium for 5 days before transfer to a Neurobasal A medium with B27 supplement containing antioxidants. No difference in the amounts of cell death between the genotypes was detected when cultures were maintained in a defined serum-free medium supplemented with antioxidants. In comparison Kim et al detected a difference in cell death rates between a PrP-null neuronal cell line and wild-type controls in serum free medium which was not supplemented with antioxidants (Kim et al., 2004). In dissociated cell culture withdrawal of serum or antioxidants is thought to lead to cell death due to sensitization to free radicals, production of which may also be elevated due to hyperoxic conditions. The failure to detect such a difference in cell death rates in the organotypic culture following serum withdrawal in the current study may be due to lower free radical production rates due to decreased oxygen availability in organotypic cultures, or due to the provision of antioxidants in B27 supplement compensating for serum-withdrawal.
PrP has been proposed to be neuroprotective via a number of different mechanisms. However, a convenient explanation centres on the reduction in SOD activity observed in PrP-null brain (Brown et al., 1997b; Klamt et al., 2001; Miele et al., 2002). This indicates reduced antioxidant capacity in PrP-null mouse brain, and therefore an increased sensitivity of PrP-null brain to further oxidative insult may underlie the observed phenotypic difference.

3.10: Variable sensitivity of hippocampal regions in PrP-null mice to organotypic cell culture:
Of the two hippocampal regions assessed within this thesis the CA1 region, but not the dentate gyrus, showed increased levels of cell death following subjection of organotypic cell cultures to a pro-oxidative environment. This leads to the question as to why the regions are differentially susceptible to degeneration.

One potential explanation is that as neurogenesis occurs in the dentate gyrus postnatally (Altman & Das, 1965), and to an age beyond that of the animals used in our experiments in rats (Kuhn et al., 1996), the dentate gyrus may be able to increase neurogenesis in response to a toxic insult thereby replacing dead/dying cells. Indeed this idea is supported by the fact that neurogenesis in the dentate gyrus has been observed to increase following damage induced by epileptic seizures (Ferland et al., 2002) and ischaemia (Bingham et al., 2005). However, a recent study has suggested that neurogenesis is not solely confined to the dentate gyrus of the hippocampus, but also occurs in the CA1, 2 and 3 regions, albeit at a considerably reduced rate relative to the dentate (Rietze et al., 2000). This indicates that the CA1 region may also be able to replace damaged neurons. However, it is possible that the variable regenerative abilities of the two regions could be responsible for the observed difference.

An alternative hypothesis is that the loss of PrP has a lesser impact upon the dentate gyrus than it does on the CA1 region. PrP is normally expressed within the dentate
gyrus (Sales et al., 1998; Moya et al., 2000) which indicates that the dentate gyrus will be subject to a loss of PrP<sup>C</sup> in PrP-null mice. However the level of expression of PrP<sup>C</sup> has been shown to be lower in the dentate gyrus than in the CA1 region (Moya et al., 2000), and this may explain why the loss of PrP<sup>C</sup> may have less of an effect within this region. Other studies have also reported that the dentate gyrus is intrinsically less susceptible to damage than the CA1 region following the loss of PrP<sup>C</sup>. Williams et al reported a lack of increase in apoptotic markers in this brain region of mice infected with 301V/VM strain of scrapie whilst the same marker was significantly increased in the CA1 region (Williams et al., 1997).

Other experimental evidence suggests that the dentate gyrus possesses a greater resistance to a number of stressors when compared to the CA1 region. These include a greater resistance to excitotoxicity (Mattson et al., 1989) and lesser calcium accumulation following ischaemia (Kubo et al., 2001). In addition it has been determined that the CA1 subfield of the hippocampus is considerably more susceptible to oxidative damage by the superoxide radical than the CA3 region (Wilde et al., 1997), and is particularly susceptible to degeneration following induction of oxidative stress (Vornov et al., 1998). Whilst it was not possible to assess neuronal death in the CA3 region of our organotypic cultures due to a lack of preservation of structure, the results of Wilde et al and Vornov et al suggest that the CA1 region of the hippocampus is intrinsically susceptible to oxidative damage by superoxide radicals. Furthermore results presented in this thesis indicate that the capacity of mitochondria to produce superoxide radicals is increased in PrP-null animals (see Chapter 5), and results reported here and in a number of published studies indicate a reduction in SOD activity (Brown et al., 1997b; Klamt et al., 2001; Miele et al., 2002) – a antioxidant enzyme which removes only superoxide.

A model can therefore be envisaged whereby the CA1 region is more susceptible to degeneration following scrapie inoculation and is particularly susceptible to superoxide-mediated oxidative damage which results in the selective degeneration of the CA1
region following an oxidative insult. Such a model provides a potential explanation for the results presented in this thesis, and also suggests that the organotypic hippocampal slice culture may provide a useful model for the study of oxidative stress in the hippocampus. In addition the retention of neuronal and synaptic morphology in organotypic slice cultures (Gahwiler et al., 1997) provides opportunities for the future study of the effects of oxidative stress upon electrophysiological activity.
Chapter 4:

*Morphology, SOD activity and respiration rates of mitochondria isolated from PrP-null mouse brain*
Background:

4.1: Investigating mitochondrial morphology, SOD activity and respiration rates in PrP-null mice:

A published study of PrP-null mice has observed altered mitochondrial morphology and altered expression of genes for mitochondrial components in brain tissue (Miele et al., 2002). However this study failed to identify any alteration in the respiration rates of cardiac mitochondria. This Chapter sets outs to determine whether indeed mitochondrial morphology and mitochondrial respiration rates are altered in PrP-null mouse brain. In order to carry out this study isolated suspensions of mitochondria prepared from brain tissue were selected as the appropriate experimental system.

Whilst mitochondrial morphological abnormalities have previously been identified in PrP-null mice (Miele et al., 2002), it is necessary to verify whether such ‘abnormal’ mitochondria were found within isolated mitochondrial suspensions. To this end suspensions of isolated mitochondria will be examined under electron microscopy and morphological assessment carried out. Furthermore the analysis of mitochondrial morphology will be extended beyond the normal/abnormal classification system employed by Miele et al to incorporate a measure of mitochondrial cristae density.

Previous reports have correlated mitochondrial morphological abnormalities with abnormalities in SOD activity in a number of disease states (Trimmer et al., 2000; McEachern et al., 2000). This fact, in conjunction with the contradictory reports regarding whether SOD activity is indeed altered in PrP-null mice (Brown et al., 1997b; Waggoner et al., 2000; Klamt et al., 2001; Miele et al., 2002; Hutter et al., 2003), justifies re-visiting this question. SOD activity in PrP-null brain tissue will therefore be assayed using a spectrophotometric NBT reduction assay.

The final section of this Chapter will address whether mitochondrial respiration rates are altered in PrP-null mouse brain tissue. Isolated mitochondrial preparations provide an ideal system for investigating mitochondrial respiration rates using a Clark Oxygen
Electrode. Manipulation of the medium bathing the mitochondria permits the measurement of respiration rates under different phosphorylating conditions and spanning various portions of the respiratory chain (see Section 4.2). This system will therefore be used to determine whether mitochondrial respiration rates are altered in PrP-null mouse brain.

4.2: Respiratory states and the respiratory chain:
The work of Chance and Williams (Chance & Williams, 1955) described 5 different respiratory steady states in isolated mitochondria. Their terminology describing these states remains in common usage. Figure 4.2.1 presents a summary diagram of the different respiratory states seen during a typical oxygen electrode experiment.

![Diagram of respiratory states](image)

*Figure 4.2.1: Representation of the result of an idealized oxygen electrode experiment demonstrating the respiratory states as defined by Chance and Williams (1955).*

The conditions to produce each respiratory state (Chance & Williams, 1955) and the physiological processes underlying each state can be summarised thus:
- State 1 respiration: mitochondria are added to the respiration medium in the absence of respiratory substrate. Oxygen consumption rates are minimal under these conditions due to the lack of substrate.
- State 2 respiration: a respiratory substrate is added to the mitochondrial suspension and a steady rate of oxygen consumption by the respiratory chain ensues. As the inner mitochondrial membrane leaks some protons, the respiratory chain operates at a level to maintain the membrane potential. Under this respiratory state the lack of a phosphate acceptor (ADP) is the rate limiting factor.
- State 3 respiration: a finite quantity of ADP is added and oxygen consumption increases. The proton gradient is dissipated through ATP synthase, disinhibiting the respiratory chain and therefore the capacity of the respiratory chain is rate limiting.
- State 4 respiration: the rate of oxygen consumption slows due to phosphorylation of all the ADP to ATP. The lack of phosphate acceptor is the rate limiting factor.
- State 5 respiration: following removal of all the oxygen from the sample (anoxia), oxygen consumption halts.
- It should be noted that State 2 and State 4 respiration are physiologically synonymous and therefore should be similar values for any given mitochondrial suspension metabolising a given substrate.
- It should also be noted that State 1 and State 5 respiration are redundant terms and values for these will not be reported within this thesis.

In addition to studying the respiratory chain under different phosphorylating conditions, it is also possible to assess respiration rates across discrete portions of the respiratory chain. This is achieved by the use of respiratory substrates that enter the respiratory chain at different points, and the use of inhibitors to block the respiratory chain at given respiratory complexes. The arrangement of the respiratory chain, the entry points of the substrates, and the respiratory complexes at which each inhibitor operates are shown in Figure 4.2.2.
The diagram of the respiratory chain shown in Figure 4.2.2 illustrates that it is possible to study discrete spans of the respiratory chain in order to identify any points of change. This thesis will study three different spans of the respiratory chain as follows:

- In order to study Complexes I, III and IV, isolated mitochondria may be provided the substrates pyruvate and malate in the absence of respiratory chain inhibitors. These substrates enter the Kreb’s Cycle and produce NADH within the mitochondrial matrix.

- In order to study Complexes II, III and IV, isolated mitochondria may be provided with succinate whilst the Complex I of the electron transport chain is inhibited with rotenone.

- To study Complex IV in isolation, the substrates TMPD and ascorbate may be provided whilst the respiratory chain is inhibited at Complex III by either rotenone, myxothiazol, antimycin A or KCN.
myxothiazol or antimycin A. In order to completely inhibit the respiratory chain
cyanide (KCN) may be added to inhibit Complex IV, which permits correction of oxygen
consumption rates for TMPD auto-oxidation.
Methods:

4.3: Preparation of mitochondrial suspensions:

Brain mitochondria were isolated according to the procedure of Lai and Clark (Lai et al., 1977), modified by using EGTA instead of EDTA. The use of a digitonin treatment suggested as by Anderson and Sims (Anderson & Sims, 2000) was investigated as a means of removing any plasma membrane enclosed structures.

Mitochondria isolated from PrP-null OLA129 male mice (Manson et al., 1994) were compared to age-matched wild-type OLA129 control animals. Animals were sacrificed by decapitation under halothane anaesthesia. The brain was rapidly dissected out and submerged in ice cold isolation buffer (0.25M sucrose, 10mM MOPS, 1mM EGTA, pH7.4). The brain was weighed prior to being minced using scissors, and then homogenized using 6 passes of a dounce homogenizer. Brain homogenate was transferred to polycarbonate centrifuge tubes (10ml per tube) and centrifuged at 2,000g for 3 minutes in a Beckman JA20 rotor at 4°C. The resultant pellet, consisting primarily of nuclear fragments, was discarded, whilst the supernatant was subjected to a further 2,000g spin for 3 minutes. The resultant supernatant was then centrifuged at 12,000g for 10 minutes to produce a crude mitochondrial pellet (P2), whilst the supernatant consisting primarily of cytoplasm and myelin was discarded.

The crude mitochondrial pellet was resuspended in 4ml 3% ficoll in isolation buffer, and layered upon 6ml 6% ficoll in isolation buffer. This discontinuous gradient was spun at 12,000g for 30 minutes. The white cloudy layer (consisting primarily of myelin) at the interface between the layers was discarded, and the pellet was resuspended in 10ml isolation buffer supplemented with 1mg/ml fatty acid free Bovine serum albumin (BSA), and with or without digitonin to a concentration of 6.25mg/g initial wet weight of tissue. Digitonin, when present, was added as a 25mg/ml stock in ethanol. After mixing, the resuspended pellet was centrifuged at 12,000g for 10 minutes. The supernatant (containing disrupted plasma membrane) was discarded and the pellet resuspended in 10ml isolation buffer with 1mg/ml BSA (fatty acid free). A further 12,000g spin for 10
minutes concentrated the mitochondrial suspension and the pellet was resuspended in 1ml isolation buffer containing 1mg BSA (fatty acid free). The BSA was added from a concentrated BSA stock solution (spectrophotometrically verified at 280nm) to optimise the accuracy of BSA concentration, enabling accurate determination of mitochondrial suspension protein concentration.

4.4: Preparation of samples for electron microscopy:
Aliquots of mitochondrial preparations from the P2 stage and following the complete isolation procedure incorporating the digitonin treatment were fixed in a 2.5% solution of glutaraldehyde in isolation buffer. Samples were fixed for 2 hours in suspension at 4°C to prevent alterations in morphology arising from samples becoming anaerobic before fixation is complete. Samples were then spun down and left in fixative overnight at 4°C. Fixed pellets were washed three times for 10 minutes each in 0.1M Millonig Phosphate buffer (150mM NaH₂PO₄ 100mM NaOH pH7.4).

All remaining sample preparation steps were carried out by Mr Stephen Mitchell (Edinburgh University School of Veterinary Studies). Post-fixation was carried out in 1% osmium tetroxide in 0.1M Millonig phosphate buffer, followed by three 10 minute washes in 0.1M Millonig Buffer. Samples were dehydrated using a graded series of solutions of acetone (50%, 70%, 90% and 100% analar grade), each dehydration step was for ten minutes and the final 100% analar grade step was repeated three times. The samples were then placed in a 50:50 araldite:acetone mixture for 30 minutes before being placed in an oven at 60°C uncovered to allow the acetone to evaporate off overnight. Infiltration was completed by three 60 minute incubations in araldite mixture, followed by two 60 minute incubations in araldite mixture with accelerator. The samples were then embedded by incubation in araldite mixture with accelerator for 48 hours at 60°C. Following complete curing, 60nm ultrathin Sections 60nm thick taken from approximately halfway through the pellet were cut using a Reichert OMU4 ultramicrotome (Leica Microsystems UK Ltd, Milton Keynes). Sections were collected on formvar/carbon copper slot grids and stained in uranyl acetate and lead citrate.

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Sections were viewed using a Philips CM12 transmission electron microscope (FEI UK Ltd, Cambridge, England) at 13,000x magnification.

4.5: Analysis of electron micrographs:
Photographic negatives were scanned at 4,800 by 9,600 dpi resolution and saved as tif files prior to analysis. Images were analysed using ImageJ software (NIH). The perimeter of the outer mitochondrial membrane was traced and defined as a region of interest to assess mitochondrial diameter, perimeter and area.

To remove the subjectivity which operates when simply categorizing mitochondria into defined groups according to cristae density, a novel technique was developed to assess cristae density quantitatively using ImageJ software. Images were background subtracted to remove any variation in background intensity which can interfere with the subsequent thresholding procedure. Image thresholding was then carried out to an extent whereby all cristae membranes were included, but no non-specific staining of the mitochondrial matrix occurred. This converts a greyscale image which is composed of pixel intensities of 0 to 255, to an image where pixel intensities are either 0 or 255 (i.e. only 2 possible pixel intensities exist) where 0 represents black areas (i.e. cristae) and 255 represents the white areas (i.e. the mitochondrial matrix). Regions of interest were then selected excluding the mitochondrial membrane. This exclusion was necessary to normalize between mitochondria of different size, as the mitochondrial membrane constitutes a different fraction of the total mitochondrial area according to the size of the mitochondrial section. The mean greyscale pixel intensity of the region of interest was recorded, which will be a Figure between 0 and 255 and is determined solely by the proportion of the selected area that is covered by cristae, 0 representing 100% cristae density and 255 representing 0% cristae density. These values were converted to a percentage to indicate the fraction of the mitochondrial interior which was made up of cristae membrane in each section (the entire cristae density analysis protocol is illustrated in Figure 4.5.1). In order to prevent the cristae density varying according to
the degree to which each sample was stained, nor according to any variations in background brightness, each mitochondria was thresholded individually. Where possible, images from wild-type and PrP-null animals were analysed sequentially to minimise any variation in the thresholding step. Only mitochondrial sections which did not show any blurring of the cristae membranes were included in the cristae density analysis, which resulted in a 27% and 29% rejection rate for wild-type and PrP-null mitochondria respectively.

Figure 4.5.1: Illustration of the cristae density analysis procedure. A – raw scanned image of one mitochondrion, scale bar = 300nm. B – subtraction of the image background results in brightening of image and simplifies thresholding procedure. C – Image is thresholded for a single mitochondrion. Limits are set so that cristae structure is obvious without any random noise being highlighted in the matrix. D – An area of interest (illustrated by the red line) is selected freehand using the inner mitochondrial membrane as a guide. E – Background subtracted image of mitochondria with bad (left) and moderate (right) blurring of the cristae, which were excluded from cristae density analysis. F – Thresholded image of E, which demonstrates lack of clarity of cristae structure (note thresholding upper limit set higher than normal to demonstrate lack of structure in blurred areas).
This technique could then be employed to compare the cristae densities between mitochondrial preparations from wild-type and PrP-null animals.

Statistics were performed using SigmaStat 2.03 as 1-way ANOVA’s with Tukey Tests to compare each mitochondrial parameter between genotypes. Statistical tests were applied to the cristae percentage density raw data, all other data was log10 transformed to conform to normality and equal variance prior to statistical testing.

4.6: Assay of brain homogenate SOD activity:
Male, age matched wild-type 129/OLA and PrP-null mice were sacrificed by halothane anaesthesia and decapitation. The skull was opened by cutting along the sagittal suture and the brain (excluding the olfactory bulb, the cerebellum and lower structures) was dissected out and immediately snap-frozen in liquid nitrogen. Brain samples were kept frozen at -80°C, and not defrosted until the day of the experiment.

Spectrophotometric determination of superoxide dismutase activity was carried out using a Nitrotetrazolium Blue (NBT) reduction assay as previously described (Oberley & Spitz, 1984).

Brain tissue was homogenized by hand, using 8 passes of a dounce homogenizer, in 1ml of 50mM sodium phosphate buffer pH7.4. This homogenate was centrifuged at 2,000g for 2 minutes and the pellet discarded to remove cell debris. The supernatant was placed into a fresh eppendorf tube and stored on ice.

Supernatant protein concentration was determined by the Lowry Assay (Lowry et al., 1951) (note that the DOC TCA solubilization-precipitation technique was not employed for these experiments as the buffer contained no interfering substances). Brain homogenates were diluted 1 in 25 for the protein assay. Following incubation of samples with Lowry Reagents A and B, absorbance at 750nm was determined
spectrophotometrically using a Smartspec 3000 (Biorad) and compared to a standard curve composed of 0.5-10mg/ml BSA standards (spectrophotometrically verified at 280nm). Protein concentrations of each sample were determined in duplicate and meaned. Following determination of sample protein concentrations, aliquots of each sample were adjusted to 0.1mg/ml protein by addition of the appropriate volume of 50mM sodium phosphate buffer pH 7.4.

A mixture comprising of 50mM sodium phosphate buffer pH 7.4, 1mM EDTA, 1mM xanthine, 56µM nitro blue tetrazolium (NBT) and 1U/ml catalase was prepared. For determination of KCN uninhibitable SOD activity this mixture was supplemented with 5mM KCN. 100µl of adjusted brain extract (giving 10µg of protein per sample) was added to 800µl of the substrate mixture and kept on ice for 20 minutes prior to commencement of the reaction, to allow KCN to maximally inhibit CuZn SOD. To initiate superoxide production, 100µl of 0.01U/ml xanthine oxidase was added to each sample, and the reaction was followed spectrophotometrically at 560nm. The SOD activity of each brain homogenate was followed in duplicate over 80 minutes for both total and KCN uninhibitable SOD. SOD activity was measured as the percentage inhibition of NBT reduction rate relative to the mean rate of control samples which contained no brain extract and were carried out in quintuplicate. Note that separate controls using KCN supplemented master mix were used during the determination of Mn SOD activity in case KCN affected xanthine oxidase activity.

4.7: Statistical analysis of SOD assay data:
Linear regressions and statistical tests were performed using Sigmaplot 2000 and Sigmapstat 2.03 (SPSS) software. As data could not be transformed to fit normality and equal variance for analysis using a 3-way ANOVA, data was tested using 2-way ANOVA’s and Tukey post-tests across genotype and animal age, but within SOD type.
4.8: Preparation of digitonin-treated and non-digitonin treated mitochondria:
As the technique of Sims and Anderson (Anderson & Sims, 2000) used rat brain rather than mouse brain and percoll rather than ficoll, it was necessary to validate the digitonin treatment protocol for use in the production of mitochondrial samples from mouse brain using the ficoll preparation technique of Lai and Clark (Lai et al., 1977). To assess the effects of digitonin, mitochondrial samples from wild-type and PrP-null mouse brain were prepared up to the digitonin addition point. Samples were then split and either treated with digitonin to a final concentration of 6.25mg/g initial tissue wet weight, or treated with an equal amount of the ethanol vehicle in which the digitonin was dissolved.

4.9: Preparation of submitochondrial particles:
Submitochondrial particles (SMPs) from whole brain were prepared by the technique of Kosenko et al (Kosenko et al., 2003) from digitonin purified mitochondrial suspensions, isolated using the procedure described previously. The final mitochondrial pellet was resuspended in 2ml lysis buffer (10mM Tris HCl pH7.4) to give a protein concentration of approximately 2mg/ml. This suspension was stirred for 15 minutes on ice, before centrifugation at 20,000g at 4°C for 30 minutes. The pellet was then resuspended in 1ml of lysis buffer and then subjected to 3 freeze-thaw cycles. Samples were snap frozen in liquid nitrogen and allowed to thaw at room temperature. The mitochondrial suspension was then transferred to thick-walled polycarbonate centrifuge tubes and spun at 144,000g at 4°C for 30 minutes using a TLA100.2 rotor in a TL-100 bench-top ultracentrifuge. Pellets were resuspended in 1ml lysis buffer and subjected to another 2 cycles of centrifugation and resuspension, with the final resuspension in 0.5ml of SMP buffer (Poderoso et al., 1996) (0.23M mannitol, 70μM sucrose, 5mM phosphate buffer, 0.2mM EDTA, 30mM Tris HCl pH7.4). SMPs were stored at -80°C and used within 3 months of preparation.

4.10: Measurement of mitochondrial oxygen consumption using an oxygen electrode:
Respiration rates of mitochondrial suspensions were monitored by following the rate of oxygen consumption in a polarographic oxygen electrode (Rank Brothers Ltd). All
experiments were conducted at 30°C to correlate with previous electrophysiological experiments conducted within our laboratory (Curtis et al., 2003) and the electrophysiological experiments reported in this thesis. Mitochondrial suspension (either 50 or 100μl) was added to 3ml of respiration buffer (25mM Sucrose, 75mM Mannitol, 95mM KCl, 20mM tris chloride pH7.4, 5mM KH₂PO₄, 50μM ethylene diamine tetraacetic acid (EDTA), 1mg/ml BSA fatty acid free), and a stable baseline acquired prior to addition of substrates.

Respiration rates through Complexes I, III and IV was measured as the oxygen consumption rate when mitochondria were provided with the Complex I substrates pyruvate and malate (final concentrations of 5 and 2.5mM respectively). Addition of rotenone to a final concentration of 8μM completely inhibited this oxygen consumption. Respiration rates through Complexes II, III and IV were measured as oxygen consumption rates when mitochondria were provided with the Complex II substrate succinate (to a final concentration of 15mM) whilst Complex I was blocked by 8μM rotenone. Oxygen consumption by succinate fuelled mitochondria could be completely inhibited by addition of myxothiazol (to a final concentration of 50nM). Complex IV respiration rates were measured as oxygen consumption rates following provision of N,N,N′,N′-tetramethylphenylenediamine (TMPD) and ascorbate (to final concentrations of 80μM and 10mM respectively), whilst Complex III was blocked by myxothiazol. To permit correction for TMPD auto-oxidation, oxygen consumption rates following complete inhibition of Complex IV with KCN were measured and subtracted from all TMPD fuelled oxygen consumption rates.

Typical experimental runs involved the acquisition of data for the different respiratory States for a single substrate entry point, whilst the respiratory chain was inhibited by an upstream inhibitor. Inhibitor concentrations were determined by titrating inhibitor until oxygen consumption, resulting from respiration fuelled by a substrate entering the respiratory chain upstream of the block point, completely ceased.
Following acquisition of a stable baseline, the State 2 respiration rate (Chance & Williams, 1955) was measured following substrate addition and prior to the introduction of ADP into the chamber. Known quantities of ADP (250-750nmoles) were added to induce State 3 respiration (Chance & Williams, 1955). State 4 respiration (Chance & Williams, 1955) was measured following the complete phosphorylation of ADP to ATP. Uncoupled respiration was measured following addition of the uncoupling protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (Fluka) to a final concentration of 1.6µM.

Respiratory control ratios were calculated as State 3 respiration rate divided by the State 4 respiration rate. ATP:O ratios were calculated as stoichiometry of ADP phosphorylation to oxygen consumption.

The respiration rates of SMP suspensions were measured in SMP buffer supplemented with 1mg/ml BSA (fatty acid free) at 30°C. SMPs were fuelled by 50mM NADH or 15mM succinate. Addition of FCCP and ADP (to final concentrations of 1.6µM and 50µM respectively) were used to determine whether SMP suspensions were coupled.

The oxygen electrode was calibrated at the beginning of each series of experiments. This was achieved by determining the oxygen electrode output in the presence of air equilibrated respiration buffer followed by complete removal of oxygen from solution by addition of excess sodium dithionite. Assuming an oxygen solubility of 7.14mg/l in a saline solution of 10ppt at 30°C, the calibration was used to express all oxygen consumption data as nmoles oxygen consumed per minute.

4.11: Determination of protein concentrations of mitochondrial suspensions:
Mitochondrial preparation protein concentration was determined using Peterson's modifications of the Lowry Assay (Peterson, 1977). This was necessary as sucrose has been demonstrated to interfere with the spectrophotometry readings. 25µl samples of
mitochondria were treated with sodium deoxycholate (DOC) to a concentration of 1% for 10 minutes to solubilise protein. Protein was precipitated by the addition of trichloroacetic acid (TCA) to a concentration of 7.2% and left at room temperature for 10 minutes. Samples were then centrifuged at 10,000g to pellet the protein and the supernatant was discarded. Pellets were resuspended in distilled H$_2$O, prior to treatment with Lowry Reagent A (copper-tartrate-carbonate, sodium dodecyl sulphate, NaOH) for 10 minutes, then Lowry Reagent B (Folin Ciocalteau phenol reagent). Colour was allowed to develop for 30 minutes before Abs$_{750}$ was measured using a Biorad SmartSpec 3000. Protein concentration was determined by a standard curve created using varying concentrations of BSA which had also been subjected to the solubilization-precipitation technique described above. Final protein concentrations were calculated by subtraction of 1mg/ml from the measured protein concentrations to counter the 1mg/ml BSA added to protect the mitochondrial suspensions. All respiration rates were normalized according to protein concentration and expressed as nmoles oxygen consumption per minute per milligram of protein.

4.12: Statistical analysis of oxygen electrode data:
Statistical testing of normalized respiration rates was performed using SigmaStat 2.03 (SPSS) software. In digitonin experiments where only a single substrate entry point was used, 1-Way ANOVA’s were performed across genotypes and across digitonin conditions, and a Tukey post-test was used to test for significance. Where multiple substrates were used to analyze the respiratory chain, 2-Way ANOVA’s were performed on the data across genotypes and substrates, but within a respiratory State (i.e. State 2, 3, 4 or FCCP uncoupled). Tukey tests were used to test for significance. When necessary all the data within a set was transformed to fit normality and equal variance to enable the application of parametric statistical tests. In the one case where it was not possible to transform the dataset to fit normality and equal variance (old animal, State 3 dataset), a non-parametric Friedman Test with a Conover Multiple Comparison test was performed using StatsDirect Software.
Results:

Electron Microscopy:

4.13: Development of a protocol to analyse mitochondrial morphology using electron microscopy:

Miele et al (Miele et al., 2002) described alterations in mitochondrial morphology in the neuropil of PrP-null mouse brain relative to wild-type controls. As any alteration in mitochondrial morphology may alter its density and hence change the fraction to which it migrates in a density gradient during isolation, an initial assessment was carried out to compare a crude mitochondrial suspension (P2) with a sample which underwent the complete isolation protocol to determine if the isolation procedure was removing any abnormal mitochondria from the PrP-null preparations. Unsurprisingly the crude preparation contained a considerable quantity of non-mitochondrial debris (see Figure 4.13.1), and a considerably greater proportion of the crude preparation was non-mitochondrial compared to the purified preparation (Figures 4.13.1 and 4.13.2). As significant differences in mitochondrial respiration rates were seen between mitochondrial suspensions from wild-type and PrP-null preparations (Sections 4.16 and 4.17), and because the purified mitochondrial preparation was highly enriched in mitochondria relative to the P2 preparation, the purified mitochondrial preparation was used for all subsequent experiments.

These results demonstrate that the ficoll gradient and digitonin treatment protocols remove substantial quantities of non-mitochondrial debris (such as synaptosomes, see Figure 4.13.1 panel B) from the preparation. As the isolation protocol utilized appeared to only affect the quantity of debris in the preparation and not the mitochondrial parameters, the use of the purified mitochondrial preparation for all subsequent experiments is validated. Electron micrographs gave consistently clear images allowing mitochondrial parameters to be analysed and compared between genotypes (Figure 4.13.2).
Figure 4.13.1: Electron micrographs of crude mitochondrial pellets prepared from wild-type (panel A) and PrP-null (panel B, scale bar represents 300nm). Considerable proportions of the structures within both preparations appear to be non-mitochondrial. (28,000x magnification)

Figure 4.13.2: Electron Micrographs of isolated mitochondria from Wild-Type (panel A) and PrP-null (Panels B and C). Panel A shows typical mitochondria from wild-type preparations showing clear structure and dense cristae, scale bar represents 300nm for all panels. Panel B shows mitochondria from PrP-null that approach similar parameters to typical wild-types. Panel C shows PrP-null mitochondria which appear abnormal when compared to controls. (13,000x magnification)
Assessment of digitized EM micrographs was performed using Image J software (NIH). Structures were identified as mitochondria by the presence of an outer membrane and by the presence of cristae (Figure 4.13.3).

Figure 4.13.3: Electron micrograph of mitochondrial preparation from a wild-type mouse. Structures labelled M were identified as mitochondria and subsequently subjected to the analysis procedures, whilst structures labelled X were identified as non-mitochondrial. Scale bar is equal to 300nm. (13,000x magnification)

Regions of interest were defined by accurately tracing the outline of the outer membrane of identified mitochondria under magnification. The area, perimeter and Feret’s Diameter of the region of interest were measured using ImageJ software (NIH). This analysis software measures Feret’s Diameter as the maximum distance between two parallel tangents to the region of interest, which is effectively the maximum diameter of the selected region.
4.14: Comparison of mitochondrial size in PrP-null and wild-type mouse brain:

The box plots shown in Figure 4.14.1 clearly demonstrate an alteration in mitochondrial morphological parameters between wild-type and PrP-null preparations. Panel A shows that the mean maximal diameter of mitochondria isolated from PrP-null animals are approximately 15% greater than wild-type controls (wild-type mean 0.306μm ± 0.004 SEM n = 496, PrP-null mean 0.352μm ± 0.005 SEM n = 435, P < 0.001). Panel B demonstrates that the perimeter of mitochondria isolated from PrP-null animals are also increased by approximately 15% relative to control animals (wild-type mean 0.839μm ± 0.010 SEM n = 496, PrP-null mean 0.975μm ± 0.013 SEM n = 435, P < 0.001). Panel C shows that the mean area of mitochondria are greater by approximately one third in PrP-null (wild-type mean 0.080μm² ± 0.002 SEM n = 496, PrP-null mean 0.106μm² ± 0.004 SEM n = 435, P < 0.001). Mitochondrial diameters were increased by approximately 15% in PrP-null animals, whilst mitochondrial perimeters were increased by approximately 15% and mitochondrial section areas by approximately 30%. This models the values predicted if most mitochondrial Sections are approximately circular in shape — a percentage increase in perimeter equal to the percentage increase in diameter, and a percentage increase in area approximately equal to the percentage increase in diameter squared. As diameter, perimeter and area all increase in this relationship, the differences are therefore due to an increase in mitochondrial size rather than altered mitochondrial section shape.

Following the observation that PrP-null mitochondria are larger than control mitochondria with respect to their diameter, perimeter and area, it is interesting to consider whether the difference is due to the presence of a finite population of abnormal mitochondria in the PrP-null sample (or indeed a population in the wild-type sample that is lost in PrP-null) or due to a general shift in mitochondrial size.
Figure 4.14.1: Box plots comparing mitochondrial parameters of PrP-null preparations (light grey) against wild-type controls (dark grey). Panel A shows data for mitochondrial diameters, panel B shows data for mitochondrial perimeter and panel C shows data for mitochondrial area. Data consists of analysis of 496 mitochondria from wild-type mice and 435 mitochondria from PrP-null mice, each approximately evenly spread across 3 mitochondrial preparations from different animals. Box plots demonstrate the median (line), 25th to 75th percentile (box), 10th to 90th percentile (bars) and 5th to 95th percentile (dots). Data underwent a log$_{10}$ transformation to conform to normality and equal variance, statistics were performed as 1-Way ANOVA's with Tukey Tests, *** representing a $P$-value of $<0.001$. 
Figure 4.14.2: Frequency plots for mitochondrial diameter (panel A), perimeter (panel B) and area (panel C) in wild-type (black bars) and PrP-null mitochondrial preparations. Data consists of analysis of 496 wild-type mitochondria and 435 PrP-null mitochondria each approximately evenly spread across 3 mitochondrial preparations from different animals. Following binning, data was normalized to a percentage of sample size to prevent differing sample sizes from interfering with data presentation. Curves were fitted to wild type (blue line) and PrP-null (red line) using Weibull 4-parameter curve fitting.

The data in Figure 4.14.2 appears to demonstrate that the curves describing mitochondrial diameter, perimeter and area are shifted to the right in PrP-null mitochondrial preparations thereby indicating increased mitochondrial size. By eye the curves show a good degree of fit to each dataset using only a single peak curve, indicating that there is no second population of ‘abnormal’ mitochondria in the PrP-null
preparation, nor a second population of abnormal mitochondria within the wild type data. The differences in mitochondrial sizes are therefore due to the spectrum of mitochondrial sizes in the PrP-null being broadened and shifted to the right.

To further ensure that the presence of an abnormal population of mitochondria within one genotype had not produced the observed difference, all data was plotted using variable bin widths (data not shown). The failure of this procedure to detect a second mitochondrial population indicates that the mitochondrial parameters within either genotype were not multimodally distributed.

Whilst these measurements all produce similar differences between the wild-type and PrP-null mitochondria, they do provide subtly different information from each other. For example the mean Feret's Diameter for the mitochondria may increase without a simultaneous increase in mitochondrial area due to an increased prevalence of longitudinal sections being included within the analysis. The differences in this variety of parameters therefore reinforces the morphological differences between the wild-type and PrP-null mitochondrial preparations and make it less likely that the differences reported are artefactual due to sampling differences, for example from mitochondria sectioned in a different plane.

The relationships between the different mitochondrial parameters were compared to determine whether the observed differences between the genotypes were caused by sampling of mitochondria of differing shape.
Figure 4.14.3: Scatter plot demonstrating the relationships between different mitochondrial size parameters for wild-type (filled circles) and PrP-null (open circles) preparations. Panel A shows the relationship between mitochondrial diameter and perimeter, panel B shows the relationship between mitochondrial diameter and area, and panel C shows the relationship between mitochondrial perimeter and area. Data consists of analysis of 496 wild-type mitochondria and 435 PrP-null mitochondria each approximately evenly spread across 3 mitochondrial preparations from different animals.

The data in Figure 4.14.3 demonstrates a close correlation between all of the mitochondrial parameters assessed, and that there is no significant change in these relationships between the genotypes. As would be predicted, mitochondrial diameter and perimeter vary linearly, whereas mitochondrial area varies with the square of the diameter and of the perimeter. The lack of alteration of these relationships with
genotype would indicate that the increase in mitochondrial size in PrP-null preparations is not due to random sampling of a significant number of mitochondria sectioned in a different plane within one genotype group. This suggests that the mitochondria in the two populations are of similar shape to each other but differ in scale. Mitochondria from PrP-null animals are more prevalent towards the right hand side of the distribution plots in Figure 4.14.2. This is unsurprising as PrP-null mitochondria were statistically significantly larger than their wild-type counterparts, and therefore were found predominantly to the right of the wild-type data points.

It is important to note that the mitochondrial pellets used to prepare EM samples may show heterogeneity with respect to mitochondrial morphology through their depth. It is, however, unlikely that the differences detected are created by sampling error from taking different portions of the mitochondrial pellet between genotypes as significant differences (at $P < 0.05$) were achieved when all samples were arbitrarily paired and statistically tested in isolation. It is also unlikely that any increase in mitochondrial size was due to any fluctuations in buffer composition or isolation procedure (resulting in swelling or shrinkage of mitochondria) as preparations from wild-type and PrP-null brain were prepared simultaneously in parallel for each experiment.

4.15: Comparison of mitochondrial cristae density in PrP-null and wild-type mouse brain:

As the cristae membranes provide the surface across which the electron transport chain operates, it was of interest to measure cristae density between the wild-type and PrP-null mitochondrial preparations.
Figure 4.15.1: Comparison of cristae densities between wild-type and PrP-null mitochondrial preparations. Panel A: Box plot showing cristae densities (expressed as percentage of mitochondrial interior) for wild-type (dark-grey) and PrP-null (light-grey) mitochondrial preparations. Data consists of analysis of 360 wild-type and 308 PrP-null mitochondria, spread approximately evenly across 3 preparations from separate animals for each genotype. Statistics were performed on raw data as One-Way ANOVA with a Tukey post-test, *** representing a P-value of <0.001. Panel B: Frequency plot of cristae densities between wild-type (black bars) and PrP-null (grey bars) mitochondrial preparations, curves fitted as modified Gaussian 4-parameter curves, blue line to wild-type data, red line to PrP-null data.

The data in Figure 4.15.1 panel A demonstrates that the cristae density of mitochondria isolated from PrP-null animals is significantly lower than in wild-type preparations, showing their cristae density is approximately 90% of that of control. Upon consideration of Figure 4.15.1 panel B the data appears to be shifted to the left in PrP-null preparations without the emergence of a discrete mitochondrial population with reduced cristae density. It therefore appears that the spectrum of mitochondrial cristae densities is broadened towards the lower density end of the spectrum in PrP-null preparations compared to wild-type, rather than there being populations of ‘normal’ and ‘abnormal’ mitochondria. Taken together, the facts that mitochondria are enlarged
(Figure 4.14.1) whilst possessing a lower cristae density (Figure 4.15.1) would tend to suggest that the mitochondria in the PrP-null are swollen relative to wild-type controls.

The cristae membranes contain the enzymes required for the ETC, and therefore the total cristae area may be indicative of the respiration capacity of a mitochondrion. The PrP-null mitochondrial preparations exhibit larger mitochondria which possess a lower cristae density when compared to control preparations. These two effects are antagonistic with respect to determining the total cristae area of a mitochondrial section. Whilst determining the cristae density, the area within each mitochondrial section was determined to allow calculation of the total cristae area within each section. The areas within each mitochondrial section were significantly greater in the PrP-null preparations when compared to the wild-type controls (data not shown), mirroring the results obtained when the mitochondrial membrane was included in the determination of the area of the mitochondrial section.

![Box plot showing cristae area per Section for wild-type (dark-grey) and PrP-null (light-grey) mitochondrial preparations. Data consists of analysis of 360 wild-type and 308 PrP-null mitochondria, spread approximately evenly across 3 preparations from separate animals for each genotype. Data was transformed using a log10 transform to make data conform to normality and equal variance, statistics were performed as a 1-Way ANOVA with a Tukey post-test, * representing a P-value of <0.05.](image)

Figure 4.15.2: Box plot showing cristae area per Section for wild-type (dark-grey) and PrP-null (light-grey) mitochondrial preparations. Data consists of analysis of 360 wild-type and 308 PrP-null mitochondria, spread approximately evenly across 3 preparations from separate animals for each genotype. Data was transformed using a log10 transform to make data conform to normality and equal variance, statistics were performed as a 1-Way ANOVA with a Tukey post-test, * representing a P-value of <0.05.
The data in Figure 4.15.2 shows that the total cristae area in mitochondrial sections from PrP-null mitochondria is significantly increased by around 10% relative to wild-type controls. This implies that whilst mitochondria from PrP-null brain tissue have their cristae more sparsely spread, their increased size results in the total cristae area per mitochondrion being actually increased relative to controls.

It is important to consider whether cristae density is proportional (either directly or inversely) to the mitochondrial size, to attempt to assess what change has occurred.

Figure 4.15.3: Scatter plot demonstrating the relationship between mitochondrial diameter and cristae density (panel A) and mitochondrial diameter and cristae area (panel B) for Wild-type (filled circles) and PrP-null (open circles) preparations. Linear regressions were applied to all data within wild-type (blue line) and PrP-null (red line) genotypes. Data consists of analysis of 360 wild-type and 308 PrP-null mitochondria, spread approximately evenly across 3 preparations from separate animals for each genotype.

The data in Figure 4.15.3 panel A demonstrates that there is no strong relationship between the mitochondrial diameter and the cristae density measured ($r^2$ for each genotype is $<$0.1). This weak correlation between mitochondrial diameter and cristae density would tend to indicate the changes in mitochondrial size and cristae density are two discrete phenomena. A tighter correlation ($r^2$ ~ 0.5) was observed between
mitochondrial size and cristae area (Figure 4.15.3 panel B). Cristae area increases with mitochondrial diameter, although the slope of this relationship in PrP-null preparations is smaller than in wild-type controls – this supports the earlier observation that cristae density is lower in PrP-null mitochondrial preparations (Figure 4.15.1).

**Superoxide Dismutase activity assay:**

4.16: SOD activity in PrP-null and wild-type mouse brain homogenate:

The data in a number of reports suggests a correlation between mitochondrial morphological abnormalities and alterations in SOD activity (Swerdlow et al., 1998; McEachern et al., 2000; Miele et al., 2002; Higgins et al., 2003; Sasaki et al., 2004). Previous studies of PrP-null mice have indicated a reduction in the activity of CuZn SOD (Brown et al., 1997b) and enhanced activity of Mn SOD (Brown et al., 1997b; Miele et al., 2002) relative to wild-type controls. A spectrophotometric determination of SOD activity was performed by following the reduction of NBT to coloured formazan product (Oberley & Spitz, 1984) to confirm these results. Two age groups of animals were tested; young animals approximately aged 1.5 months were compared with older animals of around 8-9 months in age.

The data in Table 4.16.1 demonstrates that no significant differences were detected in total SOD activity, KCN inhibitable SOD activity nor KCN uninhibitable SOD activity between the age groups within either genotype ($P>0.05$ two-way ANOVA). The data also demonstrates that total SOD activity is statistically significantly diminished PrP-null preparations when compared to wild-type controls within both the young and the aged groups, with homogenates prepared from PrP-null brain tissue inhibiting NBT reduction by approximately 90% of that of the controls. KCN uninhibitable SOD activity was significantly increased in PrP-null preparations when compared to wild-type controls, with PrP-null brain homogenates inhibiting NBT reduction approximately twice as much as controls. KCN inhibitable SOD activity was highly significantly diminished in the PrP-null preparations, which showed approximately 70% of the inhibition of NBT reduction that was observed in control preparations. If the data is
combined into single genotype groups ignoring animal age, the degree of significance (determined using 1-way ANOVA with Tukey Test) of the genotype difference for each of these measures was enhanced, with the difference between the genotypes for total and KCN uninhibitable SOD activity \( P < 0.01 \), and for KCN inhibitable SOD activity \( P < 0.001 \).

*Table 4.16.1: Comparison of total, Mn and CuZn SOD activity determined by spectrophotometric NBT reduction assay in wild-type and PrP-null male 129/OLA mouse brain.*

<table>
<thead>
<tr>
<th>% Inhibition of Formazan Product</th>
<th>Wild Type (Mean ± SEM)</th>
<th>n</th>
<th>PrP-null (Mean ± SEM)</th>
<th>n</th>
<th>( P )-value 2-Way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total SOD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young Animals</td>
<td>53.7 ± 1.3</td>
<td>10</td>
<td>48.2 ± 1.7</td>
<td>10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Aged Animals</td>
<td>55.5 ± 2.0</td>
<td>10</td>
<td>49.9 ± 1.2</td>
<td>10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>KCN Uninhibitable SOD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young Animals</td>
<td>6.8 ± 1.6</td>
<td>10</td>
<td>15.9 ± 3.9</td>
<td>10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Aged Animals</td>
<td>7.9 ± 2.7</td>
<td>10</td>
<td>16.1 ± 2.7</td>
<td>10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>KCN Inhibitable SOD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young Animals</td>
<td>46.9 ± 1.9</td>
<td>10</td>
<td>32.4 ± 4.3</td>
<td>10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Aged Animals</td>
<td>46.7 ± 3.7</td>
<td>10</td>
<td>33.8 ± 2.5</td>
<td>10</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Although no statistically significant difference in SOD activity with age was detected within either genotype, a very slight trend towards increased SOD activity was observed in the aged animals compared to the young animals. To ensure that animal age was not adversely affecting the results across genotypes, the ages of the animals within each group were compared. For the homogenate SOD assays, the young animal mean ages were Wild-Type 39 days and PrP-null 41 days (\( P > 0.05 \) unpaired \( t \)-test) and for aged animals were wild-type 261 days and PrP-null 268 days (\( P > 0.05 \) unpaired \( t \)-test). This
close age-matching for each group would suggest that animal age is unlikely to be affecting the comparison across genotypes.

As the differences between the inhibition of NBT reduction by PrP-null and control brain homogenates were not unidirectional (increase in KCN uninhibitable SOD activity and decrease in total and KCN inhibitable SOD activities), the possibility that all the differences observed are artefactual due to variable sample purity with respect to SOD can be eliminated. Additionally the protein content of the homogenates was not significantly altered between genotypes within an age group ($P > 0.05$ unpaired $t$-test) again suggesting homogenate purity is not significantly altered between genotypes.

**Measurement of Mitochondrial Respiration:**

4.17: Validation of the use of digitonin for purification of mitochondrial suspensions:

To determine the effects of digitonin treatment on mitochondrial respiration rates, oxygen consumption rates were determined in a Clark Oxygen Electrode in a number of defined respiratory states including States 3 and 4 as well as FCCP uncoupled respiration.

To assess the effects of digitonin treatment on mitochondrial respiration rates, oxygen consumption rates were determined in a Clark Oxygen Electrode in a number of defined respiratory states including states 3 and 4 as well as FCCP uncoupled respiration. As shown in Figure 4.17.1, digitonin treatment has a considerable impact on mitochondrial oxygen consumption rates following normalization to protein concentration. Protein-normalized oxygen consumption rates for State 3 and 4 respiration and FCCP uncoupled respiration were increased following digitonin treatment, the largest percentage increases being observed in State 3 respiration.
Figure 4.17.1: The effects of digitonin treatment (grey bars) versus non-digitonin treated (black bars) on State 3, 4 and FCCP uncoupled respiration of isolated mitochondria from wild-type (Panel A) and PrP-null (Panel B) OLA-129 mice. Respiration rates were measured at 30°C, and rates were normalized to protein concentration. n = 6 for all bars, with duplicate readings taken from each mitochondrial preparation. Statistics were performed as 1-way ANOVA’s with Tukey post-tests for each respiratory State. * represents P<0.05, ** represents P<0.01 and *** represents P<0.001.

The FCCP uncoupled rates represent the maximal rate of oxygen consumption by the mitochondria following addition of the pure protonophore FCCP, which can transport protons across the inner mitochondrial membrane. FCCP substantially reduces the
A consistent increase in this rate was observed for mitochondria treated with digitonin when compared to those only treated with the ethanol vehicle. As FCCP is able to equilibrate across all membranes (Nicholls & Ferguson, 2002), it will enter the mitochondria regardless of whether they are encased in a plasma membrane or not hence the rates observed in both digitonin and non-digitonin preparations represent the maximal respiratory activity of the mitochondria contained within the samples. The protein yields within the digitonin-treated samples were consistently lower than those of the vehicle-treated samples (wild-type non-digitonin 5.7mg ± 0.2, digitonin 4.3mg ± 0.3 n = 6 P < 0.05, PrP-null non-digitonin 6.2mg ± 0.3, digitonin 4.1mg. ± 0.2 n = 6 P < 0.05). As the respiration rates of mitochondrial suspensions increase following digitonin treatment and are accompanied by a decrease in the concentration of protein within the sample, it is likely that the digitonin treatment purifies the sample with respect to mitochondrial content.

State 3 respiration also represents a condition where oxygen consumption is uncoupled from the proton gradient. However it differs from FCCP uncoupled respiration as addition of ADP results in the activation of ATP synthase which phosphorylates ADP to ATP whilst dissipating the proton gradient to provide the necessary energy. The reduced proton gradient then allows the respiratory chain to run and hence consume oxygen. Thus FCCP-uncoupled and ADP-uncoupled (State 3) respiration rates represent a condition where the respiratory chain is allowed to run unhindered by the proton gradient (assuming the activity of ATP synthase is not rate limiting), but differ in their mechanism of dissipating the proton gradient. Whilst the rates of oxygen consumption between State 3 and FCCP-uncoupled mitochondria following digitonin-treatment are similar, prior to digitonin treatment the FCCP uncoupled rates are approximately 50% higher than their accompanying State 3 rate. Additionally the magnitude of the increase in respiration rate between digitonin and non-digitonin treated samples is considerably larger for State 3 than for FCCP uncoupled respiration – meaning that the increase in State 3 respiration observed following digitonin treatment cannot be purely due to a
purification of the mitochondrial suspension. Given that ADP is unable to cross the plasma membrane (Nicholls & Ferguson, 2002), and that digitonin preferentially attacks plasma membranes, the likely explanation for this result is that in the non-digitonin treated sample some mitochondria are enclosed within plasma membrane, allowing them to be uncoupled by FCCP, but not by ADP. This means that during the State 3 measurement the ADP will have no access to these plasma membrane encased mitochondria meaning they will remain coupled and thus the overall rate of oxygen consumption for the sample will be lower than compared to the FCCP-uncoupled rate. The removal of these protein-rich membranes by the action of digitonin may also explain the significantly reduced protein yields in digitonin treated samples.

State 4 respiration represents the mitochondrial oxygen consumption when the respiratory chain is coupled to the proton gradient. The rate of oxygen consumption during this state is determined by the rate at which protons leak back across the inner mitochondrial membrane. The State 4 oxygen consumption rate increased significantly after treatment with digitonin. This may be in part due to the purification of the sample, as previously mentioned, however as the increase in State 4 is greater than that seen in the FCCP-uncoupled rate in percentage terms it may be that the digitonin treatment has to some degree permeabilized the inner mitochondrial membrane which is known to be possible when using higher concentrations of digitonin (Booth & Clark, 1979).

Mitochondrial preparations are often assessed by measuring their Respiratory Control Ratio (RCR) which is calculated as the rate of State 3 oxygen consumption divided by the rate of State 4 oxygen consumption (i.e. it is the maximal rate of chain activity divided by the rate of leak). RCR’s vary according to the tissue used to prepare the mitochondria as well as the isolation technique used. In the case of mitochondria prepared from brain tissue, RCR’s of greater than 3 with pyruvate and malate as substrates are usually deemed acceptable. The technique employing the digitonin treatment outlined here routinely produced RCR’s of greater than 4 and in some cases upwards of 7. This shows that even if the digitonin-treatment resulted in some
permeabilization of the inner mitochondrial membrane, the RCR values obtained are well above the levels commonly accepted in the literature. The RCR values obtained for mitochondria not subjected to the digitonin purification step were consistently lower at around 3.5 due to the fact that proportionally State 3 increases more than the State 4 following digitonin treatment.

It is important to note that the digitonin treatment had similar effects on mitochondria isolated from both wild-type and PrP-null mice as this would suggest that any differences detected between the genotypes is not due to an artefact from digitonin treatment. As digitonin permeabilises lipid membranes, the most likely effect of digitonin treatment would be to reduce the coupling of the mitochondria. However as digitonin treatment resulted in a parallel shift (of about 50%) in the coupled rates of respiration (State 4) between Wild-type (no digitonin 12.1 nmoles/min/mg ± 0.6 SEM, digitonin 18.5 nmoles/min/mg ± 1.5 SEM, n =6) and PrP-null (no digitonin 13.1 nmoles/min/mg ± 1.0 SEM, digitonin 19.7 nmoles/min/mg ± 0.5 SEM, n=6) it can be assumed, that in isolated mitochondria there is no difference in the coupled respiration rates.

The digitonin treated mitochondria gave greater respiratory activity, vastly improved State 3 respiration and increased RCR’s. The digitonin-treatment protocol was therefore employed in all subsequent mitochondrial isolation procedures.

4.18: Oxygen Electrode analysis of respiration rates of mitochondrial suspensions prepared from aged PrP-null and wild-type control mice:

As PrP-null animals have been shown to suffer increased levels of oxidative stress (Wong et al., 2001b) which is known to increase with age, and because mitochondrial abnormalities were observed in the electron microscopy of mitochondria isolated from aged PrP-null mice (see Sections 4.14 and 4.15), a thorough investigation of the respiratory activity of these mitochondria was pursued using a Clark Oxygen Electrode.
To optimally analyse the data from the oxygen electrode, statistical analysis should be carried out within a single respiratory state between genotypes and across all the substrate entry points simultaneously in a 2-Way analysis procedure. Where the raw data or transformed raw data conformed to normality and equal variance parametric 2-Way ANOVA's with Tukey tests were used. When it proved impossible to transform data to conform to normality and equal variance less powerful non-parametric two-way analysis was carried out between genotypes across substrate entry points and within a respiratory State. One-way analysis of the data across genotypes for a single substrate entry point in a single respiratory State is also a valid analytical tool, but does not allow for any alteration in the respiratory chain to be localised to a given respiratory Complex or subset of Complexes.

![Graph](image.png)

**Figure 4.18.1:** Comparison of Oxygen Consumption rates of wild-type (black bars) and PrP-null (grey bars) isolated mitochondria during States 2, 3 and 4 respiration as well as during FCCP uncoupling. * represents $P < 0.05$
Table 4.18.1: Oxygen Consumption of mitochondrial suspensions from aged (>12 months) wild-type and PrP-null mice. Rates are expressed as nmoles oxygen per minute per milligram protein. A natural log transform was applied to all the data to make it conform to normality and equal variance for statistical testing.

<table>
<thead>
<tr>
<th>State</th>
<th>Wild Type (Mean ± SEM)</th>
<th>PrP-null (Mean ± SEM)</th>
<th>2-Way ANOVA P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pyruvate/Malate</td>
<td>11.1 ± 0.8</td>
<td>11.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>25.4 ± 1.5</td>
<td>27.9 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>TMPD/Ascorbate</td>
<td>91.0 ± 6.3</td>
<td>86.6 ± 6.0</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pyruvate/Malate</td>
<td>59.1 ± 2.1</td>
<td>68.4 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>72.1 ± 2.1</td>
<td>74.5 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>TMPD/Ascorbate</td>
<td>115.3 ± 7.2</td>
<td>111.2 ± 5.2</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pyruvate/Malate</td>
<td>12.3 ± 0.5</td>
<td>13.1 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>28.4 ± 0.7</td>
<td>30.3 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>TMPD/Ascorbate</td>
<td>103.6 ± 7.3</td>
<td>92.3 ± 7.4</td>
</tr>
<tr>
<td>FCCP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pyruvate/Malate</td>
<td>61.0 ± 1.5</td>
<td>68.1 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>69.7 ± 3.7</td>
<td>71.9 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>TMPD/Ascorbate</td>
<td>118.5 ± 8.4</td>
<td>115.2 ± 6.7</td>
</tr>
<tr>
<td>RCR</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Pyruvate/Malate</td>
<td>4.9 ± 0.3</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
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<tr>
<td>ATP:O</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Pyruvate/Malate</td>
<td>2.8 ± 0.1</td>
<td>2.8 ± 0.1</td>
</tr>
</tbody>
</table>

* unable to transform data to conform to normality & equal variance – non-parametric Friedman Test with Conover Multiple Comparison test was utilized for these points.
From the data in Table 4.18.1, there appears to be no difference in the State 4 respiration (and the physiologically synonymous State 2 respiration) between mitochondria isolated from wild-type and PrP-null mice when provided with any of the respiratory substrates utilized in these experiments. These lacks of difference in coupled oxygen consumption rates suggest that there is no change in the permeability of the inner mitochondrial membrane to protons between the two genotypes, and hence that mitochondrial efficiency is unchanged between the genotypes. However, from the data in Figure 4.18.1 and Table 4.18.1, a statistically significant increase in the FCCP uncoupled oxygen consumption rate, as well as a non-significant trend towards increased State 3 oxygen consumption rate was observed in mitochondria isolated from PrP-null OLA-129 mice relative to wild-type controls. It was not possible to perform a transformation upon the State 3 data to fit equal variance, and hence a non-parametric Friedman Test with a Conover Multiple comparison test was utilized. This non-parametric test is less powerful than parametric equivalents and may explain why although a considerable trend towards increased oxygen consumption rates in PrP-null mitochondria was observed in the State 3 data, the significance threshold of $P<0.05$ was not reached. A possible explanation for the inability to transform the data to normality and equal variance is that ATP Synthase is bordering on rate limiting and hence influenced the data. If the State 3 data for pyruvate and malate is analysed simply in a 1-Way ANOVA (across genotypes for a single substrate entry point only), significance is reached with $P < 0.01$. Overall these results represent an increase in the capacity of the respiratory chain when Complexes I, III and IV are operating with NADH produced by the Kreb’s Cycle from pyruvate and malate are fed in to the respiratory chain. As the data suggests that a difference exists between the genotypes when mitochondria were uncoupled using ADP as well as when using the pure protonophore FCCP, it can be hypothesized that the differences were not due to a change in the activity of ATP synthase between the genotypes. However, whilst this data suggests an increase in respiratory capacity at some point within the respiratory chain or Kreb’s Cycle, it does not localize it to a particular respiratory Complex.
To investigate where the observed difference may originate, substrates that enter the respiratory chain downstream of Complex I were used. No changes in State 3 or FCCP uncoupled oxygen consumption rates were observed in mitochondria when succinate (which feeds electrons into the ETC via Complex II, and onto molecular oxygen via Complexes III and IV) or reduced TMPD (which reduces cytochrome c from where electrons are passed to molecular oxygen via Complex IV) was used as substrate. This data shows a difference between genotypes when Complex I, III and IV operate, but not when Complexes II, III and IV or Complex IV alone are active. The most probable explanation for these results would be that there is a specific increase in the activity of Complex I in mitochondria isolated from PrP-null animals relative to the wild-type controls.

The respiratory control ratio (RCR) was calculated as State 3 respiration rate divided by State 4 respiration rate. Therefore the RCR can be affected by both the State 3 and State 4 respiration rates. As the data for Complex I substrates presented showed a significant increase in State 3 respiration rates without a concomitant increase in State 4 respiration rates an increase in the RCR for preparations from PrP-null animals would be predicted. Indeed this was observed with RCR’s increasing from 4.91 to 5.43 when Complex I substrates were utilized. In contrast no increase in the RCR was observed when succinate was used as a respiratory substrate. This change in RCR between the genotypes is reassuring, as RCR is not affected by the protein yield and purity of a sample with regard to mitochondrial content. Therefore it is unlikely that the differences in respiratory capacity observed are due to an artefact created during mitochondrial isolation. In order to investigate the stoichiometry of ATP phosphorylated per oxygen consumed, the ATP:O ratios were determined. No difference was seen in the ATP:O ratio between genotypes (see Table 4.18.1) suggesting that there are no alterations in the number of protons pumped or the efficiency of ATP synthase in absence of PrP.
4.19: Oxygen Electrode analysis of respiration rates of mitochondrial suspensions prepared from young adult PrP-null and wild-type control mice:
As mitochondrial morphological abnormalities and alterations in the mitochondrial respiration rates were observed between genotypes in aged animals, further experiments were undertaken to determine whether similar difference in respiration rates were evident in animals of a younger age. Therefore the complete oxygen electrode analysis of isolated mitochondrial respiration rates previously described was performed on mitochondria isolated from animals aged 3-6 months.

![Graph showing comparison of oxygen consumption rates](image)

**Figure 4.19.1:** Comparison of Oxygen Consumption rates of wild-type (black bars) and PrP-null (grey bars) isolated mitochondria during States 2, 3 and 4 respiration as well as during FCCP uncoupling from young adult mice. ** represents $P < 0.01$
Table 4.19.1: Oxygen consumption rates of mitochondrial suspensions prepared from young adult (3-6 months old) PrP-null mice and wild-type controls. Rates are expressed as nmoles oxygen per minute per milligram protein. A natural log transform was applied to the data to conform to normality and equal variance for parametric significance testing.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type (Mean ± SEM)</th>
<th>n</th>
<th>PrP-null (Mean ± SEM)</th>
<th>n</th>
<th>P-value</th>
<th>2-Way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>State 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>18.1 ± 0.9</td>
<td>22</td>
<td>17.1 ± 0.7</td>
<td>24</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>36.6 ± 3.2</td>
<td>10</td>
<td>36.1 ± 1.6</td>
<td>11</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>TMPD/Ascorbate</td>
<td>111.2 ± 7.5</td>
<td>8</td>
<td>102.3 ± 6.2</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td><strong>State 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td><strong>72.2 ± 2.1</strong></td>
<td>22</td>
<td><strong>82.3 ± 1.5</strong></td>
<td>24</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>97.9 ± 7.7</td>
<td>10</td>
<td>96.2 ± 5.9</td>
<td>11</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>TMPD/Ascorbate</td>
<td>138.2 ± 8.2</td>
<td>8</td>
<td>128.1 ± 5.2</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td><strong>State 4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>18.1 ± 0.8</td>
<td>22</td>
<td>18.7 ± 0.6</td>
<td>24</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>40.8 ± 3.1</td>
<td>10</td>
<td>43.5 ± 2.0</td>
<td>11</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>TMPD/Ascorbate</td>
<td>138.2 ± 8.2</td>
<td>8</td>
<td>128.1 ± 5.2</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
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<tr>
<td><strong>FCCP Uncoupled</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td><strong>75.2 ± 2.8</strong></td>
<td>20</td>
<td><strong>86.0 ± 2.2</strong></td>
<td>21</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>90.5 ± 6.9</td>
<td>10</td>
<td>91.1 ± 4.1</td>
<td>11</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>TMPD/Ascorbate</td>
<td>139.8 ± 7.1</td>
<td>8</td>
<td>131.6 ± 7.1</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td><strong>RCR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>4.1 ± 0.1</td>
<td>22</td>
<td>4.5 ± 0.1</td>
<td>22</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>2.5 ± 0.2</td>
<td>10</td>
<td>2.2 ± 0.2</td>
<td>10</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td><strong>ATP:O</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>2.7 ± 0.1</td>
<td>10</td>
<td>2.7 ± 0.1</td>
<td>10</td>
<td>&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>
An initial observation from the data in Table 4.19.1 is that oxygen consumption rates in mitochondria isolated from young animals are universally higher than those determined from the aged animals presented in Table 4.18.1. There are two potential explanations for this phenomenon. The first option is that there is a physiological difference between the activities of mitochondria between the two age groups, resulting from a decline in respiratory activity with age. The alternative is that the difference is an artefact created during the mitochondrial isolation procedure, whereby the preparation from the younger animals has a greater purity with regards to mitochondrial content than in the preparations from the aged animals. This second theory is supported by the fact that higher protein yields were consistently encountered in the preparations from aged animals (see Figure 4.20.1). Whilst this potential artefact remains a possibility it, however, has no negative implications towards the genotype differences observed.

The data in Table 4.19.1 allows a number of observations regarding the oxygen consumption rates of isolated mitochondria from young adult PrP-null and wild-type mice to be made. No significant differences in the rates of coupled respiration (State 2 and State 4) were observed for any of the substrates investigated, indicating that the permeability of the inner mitochondrial membrane to protons is unchanged in PrP-null mitochondria. However when rates of uncoupled respiration are considered (State 3 and FCCP uncoupled) a significant increase in the rate of oxygen consumption was observed in PrP-null mitochondria relative to wild-type controls with mitochondria provided with pyruvate and malate as substrates. A change in ATP synthase activity can be ruled out as responsible for the observed phenotype as differences in oxygen consumption were observed during both ADP and FCCP uncoupling. The differences in uncoupled respiration rates were accompanied by a trend towards an increased RCR in the PrP-null mitochondria provided with pyruvate and malate, indicating that coupled rates had not increased in parallel with the increase in coupled respiration, indicating that the difference was not a result of mitochondrial yield or purity. However when substrates enter the electron transport chain downstream of Complex I (succinate and TMPD/ascorbate) no significant differences in the rates of uncoupled rates of respiration...
were observed between the genotypes. It should be noted that, unlike in the aged animal study, a statistically significant difference was observed between the genotypes for State 3 respiration with pyruvate and malate as substrates. A possible explanation for this result is that as this dataset could be transformed to conform to normality and equal variance, the use of a powerful parametric statistical test was permitted allowing the threshold of $P < 0.05$ to be reached (in contrast to the data for aged animals). Alternatively the increased $n$ number for the young adult dataset relative to the aged animal dataset could have resulted in the detection of a significant difference between the genotypes in the young adult animal dataset alone.

These differences in mitochondrial respiration between PrP-null and wild-type controls for young adult animals mirror the results obtained from aged animals reported in Table 4.18.1. Indeed the percentage increase in uncoupled respiration rates for PrP-null mitochondrial preparations is similar between the two age groups (for young adult animals PrP-null respiration rates are 14.0 and 14.3% higher for uncoupling using ADP and FCCP respectively, and for aged animals PrP-null respiration rates are 15.7 and 11.6% higher for ADP and FCCP respectively). This indicates that the differences in uncoupled mitochondrial respiration observed do not develop during adulthood.

4.20: Potential sources of error in oxygen electrode experiments:
To validate the results obtained, some further analysis of the data were performed to ensure that the genotype differences detected were not due to experimental artefact.

Firstly the protein yields from the different preparations were compared. Protein yields in the wild-type and PrP-null mice never showed any significant difference within a single age-group (see Figure 4.20.1) negating the possibility that any of the differences observed were due to differences in yields between the genotypes. Indeed the slight trend in the young adults towards greater protein yield in PrP-null mice would tend to decrease normalized oxygen consumption rates relative to wild-type. This data, in
conjunction with the respiratory control ratio results, suggests that the differences detected are not artifacts due to mitochondrial suspension purity.

Figure 4.20.1: Protein Yields for wild-type (black bars) and PrP-null (grey bars) for both young \((n = 11)\) wild-type and \(n = 12\) PrP-null) and old animals \((n = 6)\) wild-type and PrP-null) expressed as total protein yield corrected for the addition of BSA.

As mitochondrial respiration rates were seen to decline with age (see Tables 4.18.1 and 4.19.1), it was necessary to compare the ages of the animals used in each of the genotypes within each age grouping to ensure that no significant trend in the ages of the animals used could have influenced the results. No significant differences in the ages of the animals used was observed between genotypes in either branch of the experiment (aged animals wild-type mean age 475 ± 22 days \(n=6\), PrP-null mean age 469 ± 14 days \(n=6\), young animals wild-type mean age = 129 ± 6 days \(n=11\), PrP-null mean age 132 ± 5 days \(n=12\)), and thus it is highly unlikely that animal age had any significant impact upon the results obtained within each age group.
Mitochondrial respiration rates are subject to modulation by the presence of respiratory chain inhibitors which may persist in the oxygen electrode chamber, even following a thorough washout procedure. A number of protocols were put in place to minimise any potential effect of residual traces of inhibitor in the chamber, these included: interspersing experiments on wild-type and PrP-null animals so that any build-up of residual inhibitor would affect both groups, employing an extensive wash-out routine which involved both washes with ethanol and distilled H₂O, and regularly dismantling the electrode, replacing the membrane and thoroughly washing the whole assembly. Additionally, respiration rates during FCCP uncoupling (i.e. maximal respiration rates) were plotted against experiment number (see Figure 4.20.2) to determine if any trend towards decreasing respiration rates was evident over time. As no such trend was detected this, along with the fact that respiration rates did not change following the change of membrane within an experimental set (see Figure 4.20.2 panel B), indicates that results were not affected by build up of inhibitor in the electrode chamber over time.

Figure 4.20.2: Plot of FCCP uncoupled oxygen consumption rate against experiment number for mitochondrial suspensions from wild-type (filled circles) and PrP-null (open circles). Panel A presents data for aged animal experiments, panel B presents data for the young adult experiments (Note that after experiment 17 in panel B the membrane was changed and the electrode cleaned)
4.21: Oxygen electrode studies of submitochondrial particles isolated from young adult PrP-null and wild-type control mice:

To further investigate the change that had occurred in the PrP-null preparations leading to the observed difference in mitochondrial oxygen consumption, the oxygen consumption rates of SMPs produced from PrP-null and wild-type mice were measured using the Clark Oxygen Electrode. Due to SMP yields being considerably lower than observed in mitochondrial preparations, the final reaction volume was adjusted to 2ml and data was acquired over longer time periods using lower concentrations of SMP protein. Washout of the mitochondrial matrix was confirmed by the ability of SMPs to respire aerobically when provided with NADH (to a concentration of 50mM) but not with pyruvate and malate (to concentrations of 5 and 2.5mM respectively), indicating the absence of Kreb's Cycle enzymes. The absence of any significant change in the oxygen consumption rate of SMPs upon addition of either FCCP or ADP (to concentrations of 1.6μM and 50μM respectively) indicated that SMP preparations isolated using the technique described were uncoupled.

Table 4.21.1: Oxygen consumption rates of SMP preparations from PrP-null and wild-type control mice. Rates are expressed as nmoles/min/mg protein. Typically 2 readings were taken from each SMP preparation.

<table>
<thead>
<tr>
<th>SMPs</th>
<th>Wild Type (Mean ± SEM)</th>
<th>n</th>
<th>PrP-null (Mean ± SEM)</th>
<th>n</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2-Way ANOVA</td>
</tr>
<tr>
<td>NADH Uncoupled</td>
<td>58.4 ± 3.0</td>
<td>29</td>
<td>69.7 ± 3.1</td>
<td>29</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Succinate Uncoupled</td>
<td>29.9 ± 2.5</td>
<td>29</td>
<td>32.0 ± 1.6</td>
<td>29</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Comparison of oxygen consumption rates by PrP-null and wild-type SMP preparations (Table 4.21.1) demonstrate that PrP-null SMPs have a significantly increased oxygen consumption rates (by around 20%) when provided with the Complex I substrate
NADH. However no difference in PrP-null mitochondrial oxygen consumption rates relative to wild-type controls was observed when SMPs were provided with the Complex II substrate succinate. This result agrees with the oxygen electrode data from intact mitochondria, showing an increase in respiration rates in PrP-null when provided with Complex I substrates but not when electrons enter the ETC downstream of Complex I.

The major difference between the SMP and isolated mitochondria experiments is that whilst in intact mitochondria pyruvate and malate are used to produce the reduced cofactor NADH which enters the ETC at Complex I, the absence of the Kreb’s Cycle enzymes in SMPs necessitates the use of NADH as substrate. This is possible because the inner mitochondrial membrane is inverted in SMPs thereby permitting NADH access to its binding site on Complex I. The result of this is that SMP oxygen consumption rates are unaffected by the activities of Kreb’s Cycle enzymes, thereby indicating that the difference between PrP-null and wild-type mitochondrial respiration is not a result of altered activity of the Kreb’s Cycle. In addition the fact that the matrix is washed-out of SMP preparations indicates that the difference in respiration rates between the genotypes is not the result of some soluble factor which usually resides in the matrix modulating the activity of the electron transport chain.
Discussion:

4.22: Summary and validation of the study of mitochondrial morphology of PrP-null mice using electron microscopy:

As a variety of experimental evidence suggests mitochondrial abnormality in PrP-null mouse brain, mitochondrial morphology within a suspension of isolated mitochondria was assessed under electron microscopy. As mitochondrial suspensions were prepared using a modification of the isolation procedure of Lai et al. for non-synaptic mitochondria (Lai et al., 1977), the mitochondria analysed represent a population of non-synaptic mitochondria from whole brain and therefore include both neuronal and glial mitochondria.

The study of mitochondrial morphology using electron microscopy revealed that mitochondria prepared from PrP-null animals were larger and possessed a reduced cristae density when compared to wild-type controls. The increased mitochondrial size (diameter, perimeter and area of mitochondrial sections) appeared to be caused by a skew of the distribution of mitochondrial size parameters towards larger mitochondria, rather than due to the emergence of a discrete population of abnormal mitochondria. However the total cristae area within each mitochondrial section was significantly elevated in PrP-null samples.

In order to minimize any potential artifacts due to variation in buffer osmolarity, mitochondrial preparation, sectioning or staining, PrP-null and wild-type samples were prepared in parallel. Variation in the composition of the mitochondrial suspension with regards to mitochondrial origin could reasonably be envisaged to alter the mitochondrial size parameters due to heterogeneity of mitochondria between cell types. However no discrete mitochondrial populations were observed within the study thereby suggesting that the results are not due altered sampling of different mitochondrial populations.
4.23: *Other Studies of mitochondrial morphology in PrP-null mice and TSE’s:*

One previous study has investigated mitochondrial morphology in brain tissue from the Edinburgh PrP-null mouse. Miele *et al* detected a reduced mitochondrial number and increased mean mitochondrial diameter in the neuropil of the CA1 region of the hippocampus (Miele *et al*, 2002). They also reported that an increased proportion of mitochondria in PrP-null samples were ‘non-standard’ showing ‘poorly defined and sparse cristae’. Their findings of increased mitochondrial size and decreased cristae density broadly agree with the findings presented here, although the categorization of mitochondria as normal or abnormal with regards to their cristae density is influenced by a considerably greater degree of subjectivity than the more quantitative technique used to determine cristae density within this study. The striking similarity of the results of Miele *et al* to those reported here indicate that mitochondria are morphologically abnormal throughout a significant portion of the brain in PrP-null mouse brain, rather than being an effect localized to the CA1 region of the hippocampus.

In addition to the detection of mitochondrial morphological abnormalities in PrP-null mice, the fact that abnormal mitochondria have been detected in prion disease furthers the case for mitochondrial involvement in the pathogenesis of prion disease.

Intracerebral inoculation of hamsters with the 263K scrapie agent resulted in morphological changes in the mitochondria of the cortex, cerebellum and brainstem which were evident by the time of onset of clinical symptoms. The mitochondria were found to be enlarged and to have lost their cristae (Choi *et al*, 1998), which is similar to the observations made in the Edinburgh PrP-null mouse in the current study. Similar observations have also been made in the superior colliculus of scrapie infected mice, where mitochondria have been observed to be swollen, possess a reduced cristae density and on occasion contain ‘membranous debris’ (Jeffrey *et al*, 1991). The intriguing similarities between the mitochondrial abnormalities in PrP-null animals and scrapie infected animals may indicate that the loss of PrP/C results in the development of mitochondrial morphological abnormalities in TSE’s.
4.24: Mitochondrial structural abnormalities in other neurodegenerative conditions:
Abnormalities in mitochondrial morphology are not phenomena peculiar to TSE’s. Swollen and vacuolated mitochondria possessing a reduced cristae density have been observed in cybrid cell lines created from ALS patients (Swerdlow et al., 1998) and in the G93A model of ALS (a mouse model possessing a mis-sense mutation in SOD-1 observed in some ALS sufferers) in the spinal cord (Higgins et al., 2003; Sasaki et al., 2004). These findings indicate that a similar mitochondrial structural abnormality to that observed in PrP-null mice occurs in various models of ALS. Given that some models of ALS show a mitochondrial morphological abnormality similar to that observed in PrP-null mice, it is of particular interest to note that PrP is lost in the G86R mouse model of ALS (Dupuis et al., 2002). As both PrP-null mice and ALS mouse models show similar mitochondrial morphological abnormalities, loss of copper zinc SOD activity and loss of PrPC, a relationship between PrPC expression, SOD activity and mitochondrial structural abnormality is suggested.

Mitochondrial morphology has been observed to be altered in cybrid cell lines created from patients suffering from either Parkinson’s Disease or Alzheimer’s Disease. Considerable proportions of mitochondria from cybrid cell lines appeared enlarged and possessed a decreased number of cristae (Trimmer et al., 2000).

Taken together these findings appear to suggest that the occurrence of enlarged mitochondria possessing a reduced density of cristae is common in many neurodegenerative conditions including Alzheimer’s Disease, Parkinson’s Disease and ALS as well as the transmissible spongiform encephalopathies. The observed mitochondrial abnormality therefore appears not to be a specific pathology only associated with PrP-null animals or prion disease, but a hallmark of a variety of neurodegenerative conditions.
4.25: SOD activity in PrP-null mice:

As previous reports have cited reduced total SOD activity (Brown et al., 1997b; Klamt et al., 2001), increased Mn SOD activity (Brown et al., 1997b; Miele et al., 2002) and decreased CuZn SOD activity (Brown et al., 1997b) in the Zurich and Edinburgh PrP null mice relative to wild-type controls, a SOD assay was performed to confirm this alteration in SOD activity. The results presented in this thesis are in agreement with those published by Brown et al, Klamt et al and Miele et al. However other groups have failed to detect any significant differences in SOD activity in the Zurich mouse (Waggoner et al., 2000; Hutter et al., 2003), although it has been suggested that the methodology employed by Hutter et al will remove PrPc SOD activity via usage of high concentrations of EDTA (Brown, 2005). The other major difference between the methodologies employed is the degree to which the sample is centrifuged prior to assaying the supernatant for SOD activity. Waggoner et al and Hutter et al, who have both failed to detect a difference in SOD activity, employed spins of 14,000g and 15,000g for 2x10 minutes and 30 minutes respectively whilst all those who successfully detected a difference between the genotypes employed a very gentle centrifugation step or no spin at all. This leads to the question as to whether the centrifugation steps employed by Waggoner et al and Hutter et al either removed SOD bound to some membranous structure, or whether they removed a substance capable of interacting with the assay differentially expressed in the PrP-null and wild-type mice.

4.26: Potential mechanisms underlying altered SOD activity:

The analysis of gene expression by DDRT-PCR carried out by Miele et al failed to detect any alteration in expression of either CuZn or Mn SOD (Miele et al., 2002). Whilst this result initially appears to contradict the finding that SOD activity is significantly altered in PrP-null animals, a number of credible explanations for these contradictory results can be proposed. The DDRT-PCR reaction could have failed to detect a difference in SOD transcription due to either only partial coverage of the total genome (Miele et al., 2002), or the magnitude of the change in transcription levels for SOD genes could be below the threshold for detection by the DDRT-PCR system.
utilized. Alternatively, as the SOD assays which detected a difference all detect SOD activity rather than protein levels, an alteration in SOD activity could be achieved without an increase in SOD expression. The binding of copper to CuZn SOD is essential for the enzymatic activity of the protein. Copper insertion usually occurs due to the action of an accessory protein known as copper chaperone for SOD1 (CCS) and occurs post-translationally (Culotta et al., 2006). As copper incorporation into SOD1 is regulated by copper bound CCS (Furukawa et al., 2004), the suggested role of PrP<sup>C</sup> in copper uptake (Brown et al., 1997a; Pauly & Harris, 1998) may indicate that the loss of SOD-1 activity in PrP-null mice may be due to reduction in copper availability.

An alternative mechanism whereby loss of PrP leads to decreased CuZn SOD activity has recently been proposed by Sakudo et al. An interaction between PrP<sup>C</sup> and stress-inducible protein 1 (STI1) has been demonstrated, an interaction which does not require the binding of copper to PrP<sup>C</sup> (Zanata et al., 2002). The interaction of the N-terminal half of PrP<sup>C</sup> with STI1 has been demonstrated to result in increased SOD activity (Sakudo et al., 2005b), and peptides which inhibit the interaction between PrP<sup>C</sup> and STI1 result in decreased SOD activity in PrP-expressing, but not PrP-deficient, cells (Sakudo et al., 2005a). These findings suggest that the loss of CuZn SOD activity observed in PrP-null mice may be due to the loss of the PrP<sup>C</sup> input to the STI1 signaling pathway, resulting in decreased SOD activity. However it is difficult to reconcile this model where PrP<sup>C</sup> interacts with STI1 to induce increased expression of SOD with the failure to detect an alteration in transcription of the SOD1 gene (Miele et al., 2002).

Extracellular SOD, in addition to SOD1, is sensitive to cyanide (Marklund, 1982). It is therefore not possible to exclude the possibility that a reduction in the activity of extracellular SOD enzymes is responsible for the phenotypic difference between the KCN-inhibitable SOD activities in PrP-null and wild-type mice. However the levels of extracellular SOD are considered to be relatively low in brain tissue overall, with only some subpopulations of neurons possessing EC-SOD (Oury et al., 1999; Fattman et al.,
2003). It is therefore unlikely that a reduction in EC-SOD activity could account for the considerable loss of KCN-inhibitable SOD activity observed in PrP-null mouse brain.

The binding of copper to the octapeptide repeat region of PrPc has been suggested to confer a superoxide dismutase activity to the protein (Brown et al., 1999; Wong et al., 2000), although this finding has been disputed by other groups (Hutter et al., 2003; Jones et al., 2005). It is therefore worthwhile to consider whether the loss of this putative SOD activity of PrPc could be responsible for the observed alterations in PrP-null brain homogenate SOD activities. PrPc has been suggested to confer 10% and 15% of the total SOD activity within the hippocampus and cortex respectively (Wong et al., 2000). If these values are representative of the SOD levels found across whole brain samples, the loss of total SOD activity observed within this thesis numerically agrees with the loss of PrPc-SOD being responsible for the change. However as the superoxide dismutase activity of PrPc has been shown to be KCN uninhibitable (Brown et al., 1999), PrPc SOD activity will be measured within the KCN uninhibitable fraction of SOD activity along with Mn SOD. As KCN uninhibitable SOD activity was seen to rise in the PrP-null brain homogenates relative to wild-type controls, the loss of PrPc-SOD activity cannot explain the increased KCN-uninhibitable SOD activity as the directionality of the change is incorrect. Indeed increased activity of Mn SOD appears to more than make up for the loss of PrPc SOD activity. Furthermore the fact that PrPc-SOD activity is KCN-uninhibitable means that the loss of KCN inhibitable SOD activity cannot be explained by the loss of PrPc-SOD activity. It therefore appears that a number of discrete phenomena produce the observed alterations in SOD activity – that KCN-uninhibitable SOD activity is augmented, whilst KCN-inhibitable SOD activity is reduced, and these results cannot be explained by a loss of PrPc SOD activity.

Increased Mn SOD activity in PrP-null mice may act as a compensatory mechanism for the loss of SOD-1. However the discrete localizations of CuZn (cytosolic) and Mn (mitochondrial) SOD and the low diffusability of cationic superoxide, may suggest a specific increase in SOD activity to deal with superoxide production in the
mitochondria. An alternative model is suggested by the finding that Mn SOD may be found in the cytosol during SOD-1 deficiency in fungi (Shatzman & Kosman, 1979), in a mechanism which appears to compensate for the loss of CuZn SOD. It would therefore be of interest to determine whether Mn SOD becomes delocalized from the mitochondria in PrP-null animals to compensate for reduced CuZn SOD activity.

4.27: Abnormalities of SOD activity in other conditions:
Studies of the prion protein using other models have also uncovered abnormalities in SOD activity. A significant decrease in Mn SOD activity has been observed in a mouse model which has increased expression of PrP (Hachiya et al., 2005), and decreased Mn SOD activity has been observed during scrapie infection in hamsters (Choi et al., 1998). Neither of these studies detected an alteration in CuZn SOD activity. A study of cell lines from patients suffering from sporadic ALS exhibited increased Mn SOD activity (McEachern et al., 2000), which is particularly intriguing when it is considered that PrP expression has been observed to be lost in a model of ALS (Dupuis et al., 2002). Furthermore delocalization of SOD1 to the mitochondria has been detected in mouse models of ALS, and it has been suggested that this phenomenon is causative in producing the vacuolated mitochondria often observed in ALS models (Jaarsma et al., 2001). Taken together these results may indicate a link between prion protein expression, Mn SOD activity and mitochondrial abnormality.

The importance of SOD activity can be appreciated as imbalances in SOD activity have been shown to have serious deleterious consequences. Down regulation of SOD1 has been shown to result in apoptotic cell death in neuronal cell cultures (Troy & Shelanski, 1994), mutations in SOD1 enzymes can result in amyotrophic lateral sclerosis (Barber et al., 2006) and upregulation of SOD1 has been shown to reduce apoptosis following spinal injury (Yu et al., 2006). In contrast upregulation of SOD can also be deleterious as SOD1 overexpression in conjunction with APP overexpression in trisomy 21 (Down’s Syndrome) has been proposed as a potential cause of neurodegeneration (Harris-Cerruti et al., 2004).
4.28: Summary of results of the study of mitochondrial respiration in PrP-null mice:
As the results presented in this thesis and those of Miele et al indicate mitochondrial morphological abnormalities in PrP-null mice (Miele et al., 2002), it was necessary to ensure that the mitochondrial fraction used in the oxygen electrode polarography study of mitochondrial respiration rates contained any such abnormal mitochondria. The use of an identical mitochondrial isolation protocol to that used in the electron microscopy study reported in this thesis ensured that any abnormal mitochondria were included within the fraction used for the measurement of mitochondrial respiration rates.

The oxygen consumption rate measurements presented demonstrate significant differences between mitochondria isolated from PrP-null mice relative to age and sex matched wild-type controls. When mitochondria were uncoupled by either the addition of either FCCP or ADP a significant increase in the respiration rate of approximately 15% was observed in PrP-null mitochondria when provided with Complex I substrates. This difference was not evident when substrates that enter the respiratory chain downstream of Complex I were provided. Under coupled conditions no alterations in respiration rates were detected between the genotypes for any of the substrates used, indicating that the permeability of the inner mitochondrial membrane to protons was unchanged in PrP-null animals. Overall these findings indicate an increase in respiratory capacity in PrP-null mouse brain when provided with Complex I substrates, whilst mitochondrial efficiency is unaltered.

Mitochondrial respiration rates in aged animals were significantly reduced relative to young adult animals. Whilst it is possible that this finding could be artefactual due to a decrease in mitochondrial sample purity in aged animals, similar observations have been made in a previous report which identified a reduction in respiratory capacity of synaptosomal mitochondria with age (Joyce et al., 2003). This suggests that reduced rates of respiration observed in aged animals may be due to this physiological effect. However despite the alteration in respiration rates with age, the differences between PrP-
null and wild-type mice were similar within each age group thereby suggesting that the abnormalities in PrP-null mitochondrial respiration rates do not develop with age.

4.29: Validation of the difference between PrP-null and wild-type respiration rates:
Previous investigations of PrP-null mice have indicated alterations in mitochondrial gene expression (Miele et al., 2002) and MnSOD activity (Brown et al., 1997b; Klamt et al., 2001; Miele et al., 2002), phenomena which could impact upon the mitochondrial protein content. As the respiration rates reported in this thesis are normalized to protein content, an alteration in expression of mitochondrial proteins could lead to the production of false positive results following normalization. However such an artifact would tend to alter respiration rates in all respiration states spanning all substrates and therefore is unlikely to have produced the observed Complex I specific difference. An alternative to the total protein normalization protocol employed would be the use of an assay of the activity of a specific mitochondrial enzyme such as citrate synthase. However transcription of some mitochondrial components have been shown to be altered in PrP-null mice (Miele et al., 2002), and altered enzymatic activity is possible due to differential metallation of enzymes due to the potential role of PrP in copper transport (Brown et al., 1997a; Pauly & Harris, 1998). Therefore the risk of variation between the genotypes was deemed to be greater if a single enzyme was assayed and this procedure therefore was not employed.

Miele et al have also reported altered mitochondrial number in the hippocampal neuropil (Miele et al., 2002). A number of different potential effects of this upon respiration rates measured in isolated mitochondrial suspensions can be envisaged. The simplest model is that the yield of mitochondria within the sample is decreased in PrP-nulls without affecting sample purity, and therefore the difference will be cancelled out during normalization to protein content. Alternatively a reduction in neuronal mitochondria number could result in a reduced mitochondrial suspension purity thereby depressing all respiration following normalization to protein concentration. However
not only would such an effect result in reduced respiration rates in PrP-null animals, but it would also be unable produce a Complex I specific effect.

The observed difference between PrP-null and wild-type respiration rates could theoretically be due to altered sampling mitochondrial populations from different brain regions/cell types. It would be highly coincidental, however, for the observed result to have occurred in this way as there is a high degree of similarity between the respiration rates of the mitochondrial samples from both genotypes when provided with Complex II and IV substrates, and between the protein yields in the mitochondrial samples.

Whilst an increase in the activity of the ATP synthase could theoretically result in an increase in the rate of maximal oxygen consumption by the respiratory chain, this would tend to result in an increase in respiration rates across all substrate entry points and therefore would not produce a Complex I specific effect. Additionally a difference between the genotypes is evident when mitochondria are uncoupled using the protonophore FCCP, which bypasses ATP synthase, thereby precluding the possibility that upregulation of ATP synthase was responsible for the observed difference between PrP-null and wild-type mice.

The significant alteration in the RCR indicates a non-parallel increase in uncoupled respiration relative to the coupled respiration in PrP-null preparations. This supports the idea that uncoupled respiration rates are increased in PrP-null animals as this measure is completely independent of protein yield or sample purity.

4.30: Identification of the point of change in the respiratory chain:
Whilst the results for isolated mitochondrial preparations indicate an increase in uncoupled respiration rates when provided with Complex I substrates, they do not definitively implicate Complex I activity as being responsible for the observed phenotype. In order to supply the electron transport chain of whole mitochondria with
NADH, the substrates pyruvate and malate are used and they are transported into the mitochondrial matrix where they drive the Kreb’s Cycle resulting in NADH production. The rate of NADH production is therefore dependent upon the activities of the pyruvate and malate carriers as well as the activity of the enzymes of the Kreb’s Cycle. Changes in the function of the pyruvate carrier (Halestrap, 1975) and the malate aspartate shuttle (Bremer & Davies, 1975) could alter the rate at which substrates are transported across the inner mitochondrial membrane and into the mitochondrial matrix. Upregulation of these processes could result in the increased availability of NADH. Therefore the possibility exists that the observed increase in uncoupled respiration rates for Complex I substrates in whole mitochondria of PrP-null mouse brain could be due to increased activity of Kreb’s Cycle enzymes or pyruvate and/or malate transporters. Additionally this could lead to a difference in only the uncoupled respiration rates as under coupled conditions the rate of mitochondrial oxygen consumption is primarily determined by the permeability of the inner mitochondrial membrane to protons. In addition increased calcium ion concentrations have been observed to increase the rate of glutamate, and to a lesser extent succinate, oxidation in rat heart mitochondria (Panov & Scaduto, 1995). An alteration in mitochondrial calcium buffering in PrP-null could therefore theoretically account for the increased respiration rates seen in whole mitochondria from PrP-null animals when provided with Complex I substrates due to increased activity of Kreb’s Cycle enzymes.

To determine if Complex I was the site of change in PrP-null mitochondria, the respiration rates of SMPs were measured. As the inner mitochondrial membrane is inverted or opened in submitochondrial particles, they can respire on the addition of exogenous NADH. The detection of a significant difference between PrP-null and wild-type SMP respiration rates when provided with NADH excludes the possibility that either the pyruvate transporter, the malate transporter, or the activities of Kreb’s Cycle enzymes are responsible for the observed difference between the genotypes, thereby implicating Complex I as the site responsible for the phenotypic difference.
The data from this study indicates an increase in mitochondrial respiration under uncoupled, but not coupled, conditions. The relevance of this finding can be appreciated when it is noted that the normal physiological state of mitochondria is between State 3 and State 4 and therefore increased Complex I capacity may have a physiological effect, especially under conditions requiring maximal ATP production.

4.31: Investigations of mitochondrial respiration in PrP-null animals by other groups: Three previous studies have attempted to identify altered mitochondrial respiration in PrP-null mice. All three failed to detect any significant difference between the genotypes. Miele et al (Miele et al., 2002) measured the activities of respiratory chain enzymes in myocardial tissue and revealed no significant alterations between the Edinburgh PrP-null mouse and wild-type controls. A number of features of the different tissues could explain their failure to detect a difference between PrP-null and wild-type mice, including the higher oxygen requirements of brain tissue relative to cardiac tissue, the different distribution of flux control coefficients across tissues (Rossignol et al., 2000), and the higher levels of prion protein expression in the brain (Ning et al., 2005). Brown et al (Brown et al., 2002) directly assayed the activities of respiratory complexes in brain homogenate from the Edinburgh PrP-null mouse and detected no statistically significant differences. The most likely explanation for this difference in results is that Brown et al performed their assays on brain homogenate (rather than on an isolated mitochondrial preparation) which could potentially result in modification of mitochondrial respiratory complex activity by processes such as glutathionylation (Taylor et al., 2003). Indeed Brown et al did observe a non-significant decrease in Complex I activity in the PrP-null homogenate, a result which might be expected under the pro-oxidative conditions in PrP-null mouse brain (Wong et al., 2001b). Lobao-Soares et al used the Zurich strain of PrP-null mice and performed a Clark Oxygen Electrode assay on isolated mitochondrial suspensions prepared from defined brain regions (Lobao-Soares et al., 2005) using a similar isolation procedure to ourselves (Lai et al., 1977). Although they failed to show any difference in mitochondrial respiration in any of the brain regions studied, they exclusively provided
mitochondria with succinate as a respiratory substrate and therefore crucially failed to study Complex I activity.

4.32: Studies of mitochondrial gene expression in PrP-null mice:
The phenotypic differences in mitochondrial respiration rates between PrP-null and wild-type mice reported are in broad agreement with the results of the Differential Display Reverse Transcriptase Polymerase Chain Reaction (DDRT PCR) studies of Miele et al. They reported subtle changes in the transcription of some components of the respiratory chain, including upregulation of the Complex I subunit NADH ubiquinone oxidoreductase B14.5b, along with a downregulation of Complex IV cytochrome oxidase subunit I (Miele, 2000; Miele et al., 2002). Upregulation of Complex I activity could be expected to produce the observed increase in uncoupled respiration rates when provided with Complex I substrates. However it has to be noted that Miele et al only report a small upregulation of a single subunit of Complex I. A possible explanation for this includes that the NADH ubiquinone oxidoreductase B14.5b subunit is either the rate limiting for Complex I activity or modulates Complex I activity so that its increased expression results in an overall increase of Complex I activity. However NADH dehydrogenase B14.5b is a very minor subunit of Complex I (Arizmendi et al., 1992). Alternatively other Complex I subunits may have been upregulated but remained undetected by DDRT PCR either due to the magnitude of the alteration in expression being under the threshold for detection, or because the genes responsible were out-with the coverage of that study (‘approximately 65% of the total number of genes expressed in the postnatal developing brain’) (Miele et al., 2002). The results presented in this thesis also show consistently reduced respiration rates in PrP-null animals when TMPD and ascorbate are used as respiratory substrates. Although a significant difference between the genotypes for oxygen consumption rates on Complex IV substrates is never approached, this data may hint towards a physiological effect of the reduced expression of a Complex IV subunit detected by Miele et al (Miele, 2000).
The correlation between the observed increase in respiratory capacity through Complex I in mitochondria from PrP-null mouse brain and the gene expression results of Miele et al leads to the question as to whether the upregulation of a single respiratory Complex can cause an alteration in respiration rates. Flux control analysis of mitochondrial respiration rates have demonstrated that different components of the respiratory chain are rate limiting in different tissues, and indeed Complex I is a major rate limiting component of the respiratory chain in brain tissue (Rossignol et al., 2000). It is therefore theoretically possible that increased Complex I activity may have an effect on overall respiration rates. Additionally it has also been reported that Complex I possesses a higher flux control coefficient in synaptic mitochondria (0.30) when compared to non-synaptic mitochondria (0.13) in the CA1 region of the hippocampus (Davey et al., 1997), indicating that synaptic mitochondria may be more susceptible to alterations in Complex I functionality.

However the study of gene expression in PrP-null mice detected altered expression of only a single subunit of Complex I, namely upregulation of NADH dehydrogenase B14.5b (Miele, 2000). Complex I is the least understood of the respiratory Complexes, being composed of up to 43 subunits many of which have as yet indeterminate function. Our finding that Uqcrcl (a core subunit of Complex III) overexpression in N2A cells results in increased respiration rates through Complex III (see Section 6.10) suggests that altered expression of a single subunit of a respiratory Complex has the potential to result in the alteration of electron transport rates through that Complex and therefore influence the activity of the entire respiratory chain. Therefore it is feasible that the upregulation of a single subunit of Complex I may result in an increase in respiratory activity throughout the entire electron transport chain.

NADH dehydrogenase B14.5b is a 14.5kDa protein which is not particularly hydrophobic and is found in the extra-membrane domain of the protein (Arizmendi et al., 1992). To date the function of NADH dehydrogenase B14.5b and the structurally similar NADH dehydrogenase B14.5a remains undetermined. However NADH
dehydrogenase B14.5b is a relatively small and peripheral subunit in the β region (Carroll et al., 2003) of the Complex I assembly. It therefore seems unlikely that upregulation of this single subunit would result in increased assembly of Complex I, and indeed subunit B14.5b is not suggested to play a major role in Complex I assembly in the model proposed by Ugalde et al (Ugalde et al., 2004). In addition it seems unlikely that such a peripheral subunit would be rate limiting towards electron transport through Complex I. A modulatory role towards Complex I activity seems more likely.

The studies of Miele et al also revealed subtle a down-regulation of the 16S rRNA gene (Miele, 2000). A reduction in the production of this mitochondrially encoded ribosomal component could be expected to reduce mitochondrial protein synthesis capacity. However this gene has recently been found to encode a 24 amino acid peptide known as humanin (Maximov et al., 2002). Whilst a definitive function for humanin has yet to be ascertained, the peptide has been demonstrated to protect against β-amyloid mediated toxicity (Hashimoto et al., 2001). Despite evidence that downregulation of 16S rRNA does not occur following scrapie inoculation of mice (Brown et al., 2005), alterations in 16S rRNA in PrP-null mice does suggest an intriguing link to Alzheimer’s Disease.

A number of gene array studies of mice infected with various scrapie agents (ME7, 22L, 139A and RML-Chandler) have failed to detect any significant alteration in the transcription of the genes Miele et al observed to be dysregulated in PrP-null mice (Riemer et al., 2004; Brown et al., 2005; Skinner et al., 2006). However the study of Skinner et al did detect dysregulation of a number of other mitochondrial genes, including downregulation of pyruvate dehydrogenase subunit E1 alpha and NADH dehydrogenase subunit 3 (Skinner et al., 2006), which potentially could alter rates of mitochondrial electron transport through Complex I. These findings draw into question whether the mitochondrial abnormalities observed within this study are a ubiquitous marker for transmissible spongiform encephalopathies, or are effects peculiar to PrP-null mice or the Edinburgh PrP-null mouse.
4.33: Oxidative modulation of Complex I activity:

The Complex I specific result observed could be produced by modification of Complex I activity rather than via increased amounts of Complex I protein. One mechanism of Complex I modification is via glutathionylation of thiol groups on the 51 and 75 kDa subunits of the Complex. During oxidative stress oxidized glutathione accumulates and mixed disulphides form on thiol groups of Complex I in reactions catalyzed by glutaredoxin 2 (Beer et al., 2004). Glutathionylation results in a reduction in Complex I activity accompanied by an increased rate of superoxide radical generation (Taylor et al., 2003). Increased levels of oxidative stress may therefore result in reduced respiratory activity of Complex I and induce further superoxide production. As PrP-null mice have been reported to suffer from higher levels of oxidative stress (Wong et al., 2001b), Complex I activity would be expected to be lowered in PrP-null animals. It therefore appears that oxidative modification of Complex I cannot explain the increase in respiratory activity observed in this study. Whether the chronic inhibition of Complex I by glutathionylation could lead to a compensatory mechanism operating to increase transcription of Complex I subunits and restore respiratory activity has yet to be ascertained.

The glutathionylation of Complex I is reversible (Beer et al., 2004). It is therefore possible that mixed disulphides formed on Complex I are lost during isolation of mitochondria, thereby unmasking the increased capacity of Complex I in the PrP-null animals. Whilst this would mean that the increased respiratory capacity reported here is masked in vivo, the inhibition of Complex I would result in increased levels of superoxide production by the respiratory chain (Taylor et al., 2003). This could produce a positive feedback loop leading to greater inhibition of Complex I and increased superoxide production. Indeed a recent study has uncovered signs of oxidative modification of Complex I 51kDa subunit due to production of superoxide radicals by Complex I itself (Chen et al., 2005). Studies of the exercise capacity of PrP-null animals may support this model, as PrP-nulls have been shown to possess a reduced exercise capacity relative to wild-type controls (Nico et al., 2005) which is indicative of
reduced respiratory capacity. However this potential link should be treated with extreme caution due to the multitude of different factors affecting exercise performance. An alternative view is that the removal of the mitochondrial matrix, and thereby glutaredoxin 2, in the production of SMPs could result in the degree of glutathionylation of Complex I being maintained at its physiological level in SMPs (Beer et al., 2004). However in order to produce the observed effect upon mitochondrial respiration rates, it would be necessary for Complex I to be less glutathionylated due to a higher GSH/GSSG ratio in the PrP-null animals. This appears unlikely in a mouse model known to suffer increased levels of oxidative stress (Wong et al., 2001b).

Contradictory reports suggesting oxidative stress can induce Complex I activity also exist. Oxidized lipoproteins have been observed to increase Complex I activity through a mechanism demonstrated to be mediated by oxidative stress in human umbilical vein endothelial cells (Ceaser et al., 2003). Such an induction of Complex I activity could be responsible for the observed differences in mitochondrial respiration rates as it would specifically increase respiration rates when provided with Complex I substrates and increased levels of oxidative stress have been observed in PrP-null mice (Wong et al., 2001b).

4.34: Arrangement of the respiratory chain into supercomplex assemblies:
A number of potential mechanisms could increase the rate of electron transport through the respiratory chain, including increased expression of respiratory chain components, or increased kinetics of the components of the respiratory chain. One such way in which the kinetics of the respiratory chain can be increased is via the organization of the respiratory Complexes into supercomplex assemblies which increase electron transport via electron channeling. Recently there has been considerable interest in the supramolecular organization of Complex I, with particular emphasis on it forming a supercomplex with Complex III. Evidence supporting this aggregation of Complexes I and III to form a supercomplex include kinetic evidence of similar flux control coefficients between Complexes I and III when provided with NADH (Bianchi et al.,
2004) as this indicates that the 2 Complexes operate as a single system, that large multicomplex units can be detected following a protocol to isolate membrane proteins (Schagger et al., 2004) and that Complex III is required to stabilize Complex I (Acin-Perez et al., 2004; Schagger et al., 2004). However the sets of data supporting the supercomplex assembly of the respiratory chain and the random organization of the respiratory chain have yet to be fully reconciled, and therefore it is uncertain whether the respiratory chain exists as randomly distributed single complexes, supercomplex assemblies, or a mixture of the two.

The possibility of Complexes I and III forming a supercomplex assembly could theoretically have significant impacts upon mitochondrial respiration rates via electron channeling phenomena, as the assembly of Complexes I and III into a supercomplex removes the need for ubiquinone to diffuse between distant proteins. A scheme can therefore be envisaged whereby increased assembly of Complexes I and III into supercomplex structures could result in increased uncoupled respiration rates, as was observed in the PrP-null brain mitochondrial suspensions. However the importance of lipids such as cardiolipin in the aggregation of Complexes I and III to form a supercomplex, and the loss of supercomplex assembly following lipid peroxidation (Zhang et al., 2002; Pfeiffer et al., 2003) would suggest that increased formation of supercomplexes leading to the observed increase in respiration rates on Complex I substrates in PrP-null animals is perhaps unlikely due to the increased levels of oxidative stress and lipid peroxidation in PrP-null mice (Wong et al., 2001b).

4.35: Complex I dysfunction in other neurodegenerative conditions:

A picture is emerging where the pathological effects of alterations in the functionality of the respiratory chain depend upon the respiratory control coefficient of the affected component and the energy demands of the affected tissue (Mazat et al., 1997). Brain tissue intrinsically has a high energy demand and therefore possesses a high degree of susceptibility to mitochondrial defects.
Many reports of decreased Complex I activity in neurodegenerative disease exist, of which Complex I dysfunction in Parkinson’s Disease is probably the best known. Complex I has been demonstrated to be more oxidatively damaged and possess reduced activity in Parkinson’s Disease sufferers relative to age-matched controls (Keeney et al., 2006) and known Complex I inhibitors such as rotenone produce symptoms of Parkinson’s Disease (Betarbet et al., 2000). For this reason Complex I inhibitors are used to produce animal models of Parkinson’s Disease. It has been suggested that Complex I deficiency is capable of causing the neuronal damage and cell death observed in Parkinson’s Disease (Tretter et al., 2004). However contradictory reports exist which suggest that Complex I is unaltered in Parkinson’s Disease (Hanagasi et al., 2005), although this study examined Complex I activity in platelets which possibly explains their failure to detect a difference.

Reports of increased respiratory activity in neurodegenerative conditions or models are comparatively rare relative to those of reductions in respiratory capacity. However increases in Complex I specific respiration rates have been reported in ALS. Studies of muscle biopsies from ALS patients show evidence of increased Complex I activity (Bowling et al., 1993), and more recently that patients with familial, but not sporadic, ALS possess increased Complex I and Complex II/III activity in brain tissue (Browne et al., 1998). Additionally a mouse model of ALS which possesses a SOD1 mutation has also been shown to possess elevated Complex I activity (Browne et al., 1998). However another report has cast doubt on whether respiratory activity is indeed increased in sporadic ALS skeletal muscle (Wiedemann et al., 1998), although this finding can be reconciled with the findings of Browne et al due to the different tissue utilized. Overall the suggestion of similar alterations of mitochondrial respiration in ALS and PrP-null mice, the loss of PrP in a transgenic model of ALS (Dupuis et al., 2002) and reduced SOD-1 activity in PrP-null mice suggest an intriguing link between these conditions.
4.36: Copper and mitochondrial electron transport:
The only respiratory Complex to contain copper within its structure is Complex IV – cytochrome c oxidase where the copper plays essential roles in electron transfer (Beinert et al., 1962). Copper is therefore an obvious potential link between prion protein, as it has been shown to be involved in copper uptake (Brown et al., 1997a), and respiration (Beinert et al., 1962). In comparison analysis of gene transcription has revealed a subtle down regulation of cytochrome c oxidase subunit IV (Miele et al., 2002). This result seems surprising as decreased copper availability would intuitively be expected to decrease Complex IV activity. However whilst a trend towards increased electron transport through Complex IV was observed in this study, the difference did not approach statistical significance. This casts doubt on the physiological relevance of the alteration in cytochrome c oxidase subunit I transcription, or on whether copper incorporation into Complex IV is responsible for the observed mitochondrial abnormalities.

A recent study employed the Western Blot technique to determine that levels of Complex I 15 and 39 kDa protein subunits are reduced in a neuroblastoma cell line following treatment with copper (Arciello et al., 2005). This result, taken in conjunction with our own and other findings, suggests that Complex I transcription, protein levels and activity may vary inversely with cellular copper levels. However another group has reported decreased State 3 respiration in copper deficient cardiac muscle provided with Complex I substrates (Chen et al., 2002), indicating that that the cellular response to disturbances in cellular copper concentration may result in either increased or decreased activity of Complex I. Whether these phenomena indicate a mechanism whereby Complex I synthesis is modulated directly by cellular copper concentration or by oxidative stress mediated mechanisms remains undetermined.
Chapter 5:

Assessment of superoxide production by the electron transport chain of

PrP-null mice using electron paramagnetic resonance spectroscopy
Background:

5.1: Studying mitochondrial superoxide production in PrP-null mice using electron paramagnetic resonance spectroscopy:

Published studies have reported changes in a number of parameters indicative of oxidative stress (Brown et al., 1997b; Wong et al., 2001b; Kim et al., 2004) in PrP-null mouse brain. Results presented in Chapter 4 of this thesis have indicated that PrP-null mouse brain contains structurally and functionally abnormal mitochondria. As mitochondria are the major source of free radicals in cells (Nicholls & Ferguson, 2002), this Chapter will address the question of whether mitochondrial superoxide radical production is elevated in PrP-null mice.

Oxidative stress may be assayed using a diverse array of different techniques. These include the detection of stable end-products of cellular macromolecular oxidation or measuring a biological response to oxidative damage. These techniques, however, do not directly address the question of whether superoxide production, and more specifically mitochondrial superoxide production, is altered. The technique of Electron Paramagnetic Resonance (EPR) spectroscopy enables direct detection of free radicals. However under physiological conditions the steady state concentrations of free radicals are low. This makes their measurement using EPR spectroscopy technically difficult. In order to overcome this obstacle, free radicals are stabilized by spin trapping agents prior to their detection by EPR. This study will primarily utilize the spin trap tempone-H which has previously been demonstrated to display promising characteristics (Dikalov et al., 1997a).

The aim of this Chapter is therefore to develop a technique which enables mitochondrial superoxide production to be assayed using EPR spectroscopy and tempone-H spin trapping. If successful, such a technique would then be employed to investigate whether the rate of superoxide production by the respiratory chain is altered in PrP-null mice.
Methods:

5.2: Detection and analysis of EPR spectra:

All EPR recordings were made using a Magnettech MS100 bench-top EPR spectrometer. EPR Spectrometer settings used for acquisition of spectra from the oxidation of hydroxylamine spin traps (tempone-H and CP-H) were based on the techniques of Dikalov et al. (Dikalov et al., 1997b), incorporating a reduced sweep time to increase temporal resolution. Spectrometer settings were centre field 3365.8G, sweep width 51.2G, sweep time 20 seconds, sweep number 3, modulation amplitude 1500mG, microwave power 20mW (power attenuation 7), and an appropriate signal gain to detect the EPR spectrum at high resolution. For experiments involving the spin trap DMPO EPR spectrometer parameters used to acquire spectra were: centre field 3369.6, sweep width 98.7, sweep time 60 seconds, sweep number 5, modulation amplitude 1500mG, microwave power 50mW and receiver gain set at maximum.

To assess the spin signals from aqueous samples or suspensions, samples were sealed in 50μl calibrated glass capillaries (Camlab) using Critaseal capillary sealing compound (Fisher). Batches of capillaries were tested for consistency using tempone (Alexis Biochemicals) standards.

In order to ensure data was unaffected by contamination of the holding tube due to leakage from the capillaries, the spectrometer was regularly tested without a capillary in place. If clear EPR absorption peaks were observable under these conditions, the holding tube was replaced.

Data was acquired using Miniscope Control Software (Magnettech). Analysis was performed upon either peak to peak amplitude or area under the curve of the EPR spectra acquired using Magnettech Analysis Software (see Appendix Section A2.1). The peak to peak analysis technique was used for routine analysis of spectra. Graphs and linear regressions were plotted using Sigmaplot 2000 (SPSS), statistical analysis was performed using SigmaStat 2.03 (SPSS) software. When required, control spectra
were subtracted from experimental data by waveform subtraction using Magnettech Analysis software (see Appendix Section A2.2).

5.3: Detection of tempone EPR signals:
Spectra acquired from solutions of the free radical tempone (Alexis Biochemicals) of varying concentrations (1μM to 1mM) dissolved in phosphate buffered potassium salt (PBKCl) (120mM KCl, 30mM sodium phosphate buffer, pH7.4) were used to make initial assessments of spectrometer sensitivity and validity of analysis procedures.

5.4: Auto-Oxidation Reactions:
Hydroxylamine spin traps such as tempone-H are subject to auto-oxidation reactions in aqueous solution due to the presence of contaminating transition metals (Dikalov et al., 1999). As this leads to the generation of significant background EPR signals, techniques were put in place to minimize transition metal contamination and thereby reduce tempone-H auto-oxidation rates.

Transition metal contamination was reduced by the use of the ion exchange resin Chelex-100 (Sigma) which selectively replaced contaminating transition metal ions with sodium ions. Reagents and buffer solutions were treated with Chelex-100 resin to a concentration of 0.05g/ml for a minimum of 6 hours at 4°C before use in any EPR experiment. Following chelex treatment the solution pH was checked and adjusted to 7.4 by drop-wise addition of NaOH or KOH where necessary.

To minimize transition metal contamination introduced in enzyme stocks, all enzyme stock solutions were dialysed against the appropriate chelexed buffer three times using microdialysers (Triple Red UK). Cellu Sep cellulose dialysis membranes (Membrane Filtration Products Inc.) of nominal thickness 28μm and 3,500 Molecular Weight Cut-Off (MWCO) were used in dialysis protocols.
An established protocol (Membrane Filtration Products Inc.) was used to remove contaminating heavy metals from dialysis membranes. Membranes were cut into sections approximately 2cm², soaked in distilled water for 15 minutes, transferred to 10mM sodium bicarbonate and kept at 80°C under constant stirring for 30 minutes. Dialysis membranes were then immersed in 10mM EDTA at room temperature for 30 minutes, before the EDTA was replaced with distilled water and heated to 80°C under constant stirring for 30 minutes. Dialysis membranes were batch prepared and stored under 50% ethanol. Residual ethanol was rinsed off membranes prior to their use in dialysis experiments.

The ability of the chelex treatment and dialysis techniques to reduce transition metal contamination was assessed by following the development of a tempone EPR signal derived from the auto-oxidation of 1mM tempone-H (Alexis Biochemicals) in PBKCl (see Appendix Section A3.1). Residual auto-oxidation of tempone-H was subtracted off-line by waveform subtraction of the appropriate control sample (see Appendix Section A2.2).

5.5: Production of tempone spin signals:
The ability to detect the superoxide-mediated oxidation of tempone-H to tempone using EPR spectroscopy was determined to validate the technique. Assessment of tempone production from tempone-H oxidation was carried out using two different sources of superoxide radicals. Firstly incubation of 1mM tempone-H with 20mM potassium superoxide (KO₂) for 4 hours was used to verify the superoxide mediated oxidation of tempone-H to tempone. And secondly superoxide production by a xanthine/xanthine oxidase (Sigma) couple (0.5mM and 0.0005U/ml respectively) was followed at 30°C by EPR spectroscopy using 1mM tempone-H as spin trap. This xanthine/xanthine oxidase system produces superoxide radicals at a constant rate over a period of time, which more closely mimics the biological production of free radicals than KO₂ addition. The xanthine/xanthine oxidase couple is known to produce both superoxide and hydrogen peroxide (Hodges et al., 2000) in the reactions:
Xanthine + H₂O + O₂ → Uric Acid + H₂O₂
Xanthine + H₂O + 2O₂ → Uric Acid + 2H⁺ + 2O₂⁻

In order to determine whether superoxide or hydrogen peroxide production resulted in the generation of the detected EPR signal, each species was removed by the addition of dialysed CuZn SOD to a final concentration of 20U/ml and dialysed catalase to a final concentration of 100U/ml respectively.

To determine the relationship between tempone-H concentration and the proportion of superoxide trapped, superoxide dependent tempone production was assessed by EPR using tempone-H concentrations in the range of 0.5-2.0mM. Tempone EPR signals were considered to be experimentally generated following subtraction of an auto-oxidation control for the appropriate tempone-H concentration.

5.6: Potential cross-reactions:
Previous reports have indicated that tempone is subject to reduction by a number of biological antioxidants (Dikalov et al., 1997b) which could interfere with the assessment of superoxide production using this spin trap. Therefore the sensitivity of tempone solutions to reduction by the biological antioxidants ascorbate (Sigma) and reduced glutathione (Sigma) were assayed by EPR spectroscopy using variable concentrations of tempone and antioxidant at different temperatures (see Appendix Sections A4.1 and A4.2).

The alternative hydroxylamine spin trap CP-H (Alexis Biochemicals) and its oxidized form 3-CP (Sigma) were investigated as potential alternatives to tempone-H/tempone as they have been shown to exhibit increased resistance to biological antioxidants (Dikalov et al., 1997b) (see Appendix Section A5.2).
5.7: Calibration Reaction:
To allow a comparison of superoxide measurement by tempone-H spin trapping and EPR spectroscopy to established techniques, quantities of superoxide generated by a xanthine/xanthine oxidase couple were measured in parallel by both EPR and spectrophotometric detection of ferricytochrome c reduction. EPR detection of superoxide production was achieved by incubation of xanthine/xanthine oxidase with tempone-H to a final concentration of 1mM.

Superoxide detection using the Spectrophotometric technique of Fridovich (Fridovich, 1970) was carried out by following the reduction of ferricytochrome c (Sigma) to ferocytchrome c (reaction shown below) at 550nm on a Biorad Smartspec 3000. Ferricytochrome c concentrations used were in the range 5-40μM. Superoxide concentrations were calculated by linear regression of the double reciprocal plot of absorbance against ferricytochrome c concentration to determine the y-intercept. This was converted to a superoxide concentration using a published molar extinction coefficient of 2.1 x 10^4 M/cm (Massey, 1959).

\[ \text{O}_2^{•-} + \text{Ferricytochrome c} \rightarrow \text{O}_2 + \text{Ferocytchrome c} \]

Superoxide dependence of signals in both assays was checked by addition of 20U/ml dialysed CuZn SOD.

5.8: Preparation of isolated mitochondria and submitochondrial particles:
Mitochondria were prepared from mouse brain using the techniques described in Section 4.3 employing the digitonin purification protocol. SMPs were prepared from mitochondria using the techniques described in Section 4.9.
5.9: Respiration rates of isolated mitochondria in the presence of hydroxylamine spin-traps:
The respiration rates of isolated mitochondria from PrP-null and wild-type control mice were determined using a Clark Oxygen Electrode as described in Section 4.8. To determine if the spin trap tempone-H had any discernable effect upon mitochondrial respiration rates, oxygen consumption rates of mitochondria provided with pyruvate/malate were determined for respiratory states 2, 3 and 4 and FCCP uncoupled respiration both in the presence and absence of the spin trap. Experiments were carried out in both the presence and absence of Tempone-H within each mitochondrial preparation. Tempone-H and tempone, when present, were added to final concentrations of 1mM.

5.10: Investigation of mitochondria using electron paramagnetic resonance spectroscopy and tempone-H:
To assess the feasibility of detecting superoxide production by intact mitochondria using tempone-H spin trapping and EPR, mitochondrial suspension (approximately 5mg/ml protein) was diluted 1 in 5 into chelexed respiration buffer supplemented with 1mM tempone-H, 5mM pyruvate and 2.5mM malate and incubated at 30°C. 50μl samples were taken at regular intervals for measurement of their EPR spectrum. Control samples lacking mitochondria, but including the mitochondrial buffer, were run in parallel to permit correction for auto-oxidation of tempone-H.

5.11: Assessment of superoxide production and antioxidant capacity of submitochondrial particles using tempone-H and tempone:
To investigate whether any component of the SMP suspension was capable of reducing tempone, quantities of SMP suspension (adjusted to make protein concentrations equal to those used in intact mitochondria experiments) were incubated with tempone to a concentration of 500μM at 30°C.
To determine the feasibility of detecting superoxide production by the respiratory chain in SMPs using EPR and tempone-H spin trapping, SMP suspension was diluted into chelexed SMP buffer (0.23M mannitol, 70μM sucrose, 5mM phosphate buffer, 0.2mM EDTA, 30mM tris chloride, pH 7.4), containing 1mM tempone-H (final protein concentration of 0.5-0.8mg/ml). The SMP/buffer/tempone-H mixture was kept on ice for 30 minutes prior to each experiment to allow equilibration of tempone-H into SMPs. Complete blockade of the respiratory chain by all inhibitor concentrations utilized was verified for each SMP preparations by complete abolition of measurable oxygen consumption in a Clark oxygen electrode. To assess superoxide production from the respiratory chain Complexes I, III and IV, NADH to a final concentration of 1mM was added as substrate. To assess superoxide production from Complexes II, III and IV, succinate (15mM) was added in the presence of 8μM rotenone to block ETC Complex I. 8μM rotenone, 50nM myxothiazol, 18μM antimycin A and 1mM KCN were used to inhibit Complexes I, III, III and IV respectively. The rate of superoxide production by the electron transport chain was measured as the increase in the rate of tempone formation following blockade of the respiratory chain. Superoxide dependence of signals was verified by inhibiting the signals via addition of dialysed CuZn SOD to a final concentration of 20U/ml. In order to minimize variation of auto-oxidation and SMP concentrations within experiments, single tempone-H stocks were used for each experiment and mixed with SMP suspension before aliquoting and addition of substrates and inhibitors.

5.12: EPR investigation of mitochondrial antioxidant capacity using electron paramagnetic resonance spectroscopy and tempone reduction:

To assess the ability of mitochondrial antioxidants to reduce tempone, mitochondrial suspension (to a final protein concentration of approx 4mg/ml) was incubated at 37°C with 500μM tempone. Assessment of tempone reduction by antioxidants was carried out in 50μl samples using EPR spectroscopy. A temperature of 37°C was selected for
these experiments to minimize any lag prior to reduction of tempone by mitochondrial antioxidants (see Figure 5.16.3).

In an attempt to identify which antioxidant species could reduce tempone to an EPR-silent compound, removal of antioxidants using hydrogen peroxide, and 1-chloro-2,4-dinitrobenzene (CDNB) was carried out. Hydrogen peroxide non-selectively oxidizes antioxidant compounds, whereas CDNB specifically removes reduced glutathione via conjugation reactions (Habig et al., 1974). Hydrogen peroxide treatment was carried out by addition of hydrogen peroxide to a final concentration of 0.9%, samples were kept on ice for 5 minutes and, where stated, were centrifuged at 12,000g for 5 minutes and then resuspended in fresh buffer to remove residual hydrogen peroxide. Treatment with CDNB was achieved by incubating mitochondrial suspensions diluted to approximately 2.5mg/ml protein with 1mM CDNB. Samples were incubated at 37°C for 2 minutes, centrifuged at 12,000g and then resuspended in mitochondrial isolation buffer.
Results:
5.13: Tempone EPR signal generation from tempone-H oxidation:
To confirm that the spin trap tempone-H could be converted by superoxide to tempone (producing a spin signature), known concentrations of tempone-H were treated with 20mM KO$_2$ for 4 hours prior to EPR signal detection.

![Figure 5.13.1: Comparison of the EPR signal intensities obtained from varying concentrations of tempone (filled circles) and from varying concentrations of tempone-H treated with 20mM KO$_2$ for 4 hours (open circles). Spectra represent data from 100μM tempone (black) and 100μM tempone-H treated with KO$_2$ (red). Tempone/tempone-H was dissolved in a buffer composed of 50mM NaOH and 1mM EDTA disodium salt. n=3. Linear regressions were applied to all data for tempone (blue line) and tempone-H (red line) datasets.](image)

No significant differences were detected in the relationships between tempone/tempone-H concentration and EPR signal amplitude, as shown in Figure 5.13.1. This verifies that tempone-H can be converted to a compound which shows tempone spin signature. In addition, as the signals are proportional to each other at a ratio of 1:1, the stoichiometry...
of the reaction is confirmed as one mole of tempone-H converted to one mole of tempone as predicted by the reaction:

$$\text{Tempone-H} + \text{O}_2^- \rightarrow \text{Tempone} + \text{H}_2\text{O}_2$$ (Dikalov et al., 1999)

The close correlation of the signal magnitudes from oxidized tempone-H and tempone also suggests that the purities of the tempone-H and tempone used were similar.

5.14: Detection of EPR spectra:
The concentration of radical species can be determined by the intensity of their EPR spectra. Tempone standards were used to assess the sensitivity of the EPR setup to tempone.

![Figure 5.14.1](image)

Figure 5.14.1: ESR spectra of varying concentrations of tempone in PBKCl pH7.4, incubated at 30°C for 10 minutes. Spectrometer settings were as stated in methods section.

Figure 5.14.1 demonstrates that clearly observable peaks are produced when tempone is present in micromolar concentrations and the EPR spectrometer is setup as described in Section 5.2. The spectra in Figure 5.14.1 also show that the amplitude of the waveform increases with tempone concentration, whilst the peak width remains constant.
To determine the relationship between tempone concentration and EPR signal magnitude, a concentration response curve was constructed over a range of 0 to 100µM tempone, and spectra were assessed according to the peak-peak amplitude analysis technique (see Appendix Section A2.1). Figure 5.14.2 panel A shows that, in a simple phosphate buffered salt solution incubated at 30°C, there is no significant change in EPR signal intensity over a ninety minute time period. This indicates that tempone is stable in solution under these conditions. The implication of this is that the duration for which tempone has been in solution does not affect its EPR signal and therefore tempone EPR signals may accumulate in a linear fashion under experimental conditions. Panel B plots the 10 minute incubation data from panel A against the tempone concentration. A linear relationship ($r^2 > 0.99$) between tempone concentration and EPR signal intensity was identified. Further experiments (data not shown) demonstrated that the linear relationship between tempone concentration and EPR signal intensity held true up to tempone concentrations of at least 1mM.

EPR signal amplitude is therefore directly proportional to tempone concentration within the range 0-1mM tempone when measured using the techniques described. In order to account for any fluctuations in the EPR spectrometer over time, similar tempone calibration curves were constructed at regular intervals and used to convert data to tempone concentrations where appropriate.
Figure 5.14.2: EPR signals from tempone in PBKCl pH 7.4, incubated at 30°C. Panel A: Stability of tempone EPR signal over time at different tempone concentrations. Error bars represent SEM, linear regressions applied to all data at each tempone concentration, n = 3. Panel B: The relationship between tempone concentration and EPR signal amplitude following 10 minutes incubation at 30°C. Error bars represent SEM, n = 3. Linear regression applied to all data, gradient = 237.1, y-intercept = 19.8, \( r^2 = 0.995 \).

Whilst KO₂ can be used to generate large quantities of superoxide almost instantly, it is less useful for mimicking free radical production by mitochondria which occurs at low levels over prolonged periods. The xanthine/xanthine oxidase system was selected as a suitable system for the production of superoxide at slow controllable rates.
Figure 5.14.3: EPR spectra obtained from solutions containing 1mM tempone-H and various components of the xanthine/xanthine oxidase system following 50 minutes of incubation at 30°C. Xanthine concentration 0.5mM, xanthine oxidase concentration 0.001U/ml, SOD concentration 50U/ml and catalase concentration 100U/ml.

The EPR spectra shown in Figure 5.14.3 demonstrate that small tempone EPR signals were produced when 1mM tempone-H was incubated with either xanthine or xanthine oxidase. In comparison when 1mM tempone-H was incubated with both xanthine and xanthine oxidase an EPR signal of considerably greater magnitude was produced. As the EPR signal in the presence of xanthine and xanthine oxidase is greater than the summated auto-oxidation rates of tempone-H in the presence of the individual components of the enzyme/substrate couple (by waveform subtraction of the summated xanthine and xanthine oxidase controls), the tempone EPR was dependent upon a product of the xanthine/xanthine oxidase reaction.
Figure 5.14.4: Time-course of tempone EPR signal evolution from a xanthine/xanthine oxidase superoxide generation system. All tubes contained 1mM tempone-H in Chelex treated phosphate buffered saline, and where present xanthine (X) to a concentration of 0.5mM, dialysed xanthine oxidase (XO) to a concentration of 0.001U/ml, dialysed SOD to a concentration of 50U/ml and dialysed catalase (cat) to a concentration of 100U/ml. Error bars represent SEM, n = 3.

The data in Figures 5.14.4 demonstrates that EPR can be used to detect superoxide generated by xanthine/xanthine oxidase when 1mM tempone-H is used as a spin trap. When the complete xanthine/xanthine oxidase superoxide generation system is present tempone signals are approximately 10-15 times greater than when xanthine or xanthine oxidase are present alone, indicating that a product of the enzyme substrate couple (rather than summation of auto-oxidation rates) is responsible for the tempone signal generation. As the xanthine/xanthine oxidase couple is known to produce both
superoxide radicals and hydrogen peroxide (Hodges et al., 2000), the enzymes SOD and catalase were used to assign the tempone signal to a particular reactive oxygen species. Addition of dialysed CuZn SOD abolished the tempone signal to control levels, whilst catalase had no significant effect upon tempone signal evolution. This allows the tempone signal to be assigned to superoxide generation by the xanthine/xanthine oxidase couple. The declining rate of tempone signal evolution in the xanthine/xanthine oxidase samples seen over time is presumably due to inactivation of the enzyme by hydrogen peroxide (Green & O'Brien, 1967).

The rate of tempone signal evolution was lowest in the SOD control samples. A potential explanation for this is that SOD partially inhibits tempone formation from auto-oxidation reactions, as 50% of auto-oxidation is superoxide mediated (Dikalov et al., 1999).

Superoxide may be lost from solution via dismutation or via its reaction with other molecules in solution or suspension. These reactions compete with tempone-H for superoxide, and may result in only a finite proportion of the superoxide generated by the xanthine/xanthine oxidase system reacting with the spin-trap. This would result in the tempone concentration (and hence tempone EPR signal magnitude) being lower than the total quantity of superoxide produced, and therefore underestimation of superoxide production rates. To determine the proportion of superoxide generated by xanthine/xanthine oxidase which is trapped by tempone-H, a range of tempone-H concentrations 0.5 to 2.0mM were incubated with the xanthine/xanthine oxidase superoxide generation system with no competing superoxide scavenging compounds.
Figure 5.14.5: The effects of tempone-H concentration upon superoxide dependent tempone EPR signal generation. Xanthine concentration 0.5mM, xanthine oxidase concentration 0.0005U/ml, SOD concentration 50U/ml, n = 3. Panel A demonstrates the variable auto-oxidation rates of 0.5 (open triangle), 1.0 (filled triangles), 1.5 (open circles) and 2.0mM (filled circles) tempone-H concentrations. To control for these variable auto-oxidation rates, appropriate auto-oxidation controls were subtracted from the data in Panels B and C. Panel B shows a plot of the development of an EPR signal over time at different concentrations of tempone-H. Panel C shows a plot of the effect of tempone-H concentration upon EPR signal intensity after given periods using identical superoxide generation systems. Linear regressions were applied to all data at a single time-point to demonstrate the relationship between superoxide-dependent tempone EPR signal development.
As the auto-oxidation rate varies with tempone-H concentration (see Figure 5.14.5 panel A), a control was carried out for each tempone-H concentration. From Figure 5.14.5 panels B and C it can be seen that the EPR signal intensity, following correction for auto-oxidation, does not vary with tempone-H concentration between 0.5 and 2.0mM when rates of superoxide generation are approximately 0.1μMmin⁻¹. This suggests that under these conditions the superoxide is completely trapped by 1mM tempone-H, and indeed 0.5mM tempone-H is sufficient for complete trapping. However it must be noted that the rate of spontaneous superoxide dismutation is second order with respect to superoxide concentration, whilst the reaction with tempone-H is first order with respect to superoxide concentration. This means that as superoxide concentration increases, the increase spontaneous dismutation rate will be proportionally greater than the rate of reaction with tempone-H, thereby potentially resulting in only partial trapping of superoxide by tempone-H. These results would suggest that if superoxide generation rates are lower than those used in this experiment and 1mM tempone-H is present then virtually all superoxide will react with tempone-H. It is also important to note that as 0.5mM was sufficient to trap all superoxide in the reaction, the reduction of tempone-H concentration to as low as 0.5mM, due to either compartmentalization effects or inaccuracy in tempone-H stock preparation, is unlikely to adversely affect any data assuming appropriate auto-oxidation controls are in place.

5.15: Calibration of superoxide detection by tempone-H spin trapping and electron paramagnetic resonance spectroscopy:

To assess the ability of tempone-H spin trapping and EPR spectroscopy to quantitatively assess superoxide production, superoxide produced by a xanthine/xanthine oxidase couple was measured by EPR. A parallel experiment measured superoxide production by an identical system by following the reduction of cytochrome c using spectrophotometry, thereby permitting comparison of the techniques.

Figure 5.15.1 displays data from one experiment determining superoxide concentration by spectrophotometric detection of ferricytochrome c reduction. The data demonstrates
a good degree of fit for the double reciprocal plot. This indicates the assay method as performed should give a good estimate of superoxide production.

Figure 5.15.1: Example cytochrome c data from a single experiment with incubations for 50, 60 and 70 minutes. Panel A is raw data plot of superoxide dependent absorbance (subtracted for a xanthine oxidase control) against ferricytochrome c conc. Panel B shows the double reciprocal plot used to determine maximal absorbance. Max absorbance was calculated by linear regression of the double reciprocal to determine the y-intercept.
Figure 5.15.2: Scatter plots demonstrating the correlation between superoxide concentrations measured using ferricytochrome c and by EPR utilizing tempone-H spin trapping. Panel A shows EPR data that has been control subtracted for a xanthine oxidase control, panel B shows EPR data that has been subtracted for a xanthine/xanthine oxidase/SOD control. Data was acquired at set time-points using both techniques, and the experiments were carried out on 10 separate occasions. Panel C overlays data from tempone standards (open circles, red regression line) over the data from Panel A (filled circles, blue regression line).
The data in Figure 5.15.2 panels A and B demonstrate the existence of a linear relationship between the concentration of superoxide detected by spectrophotometric determination of cytochrome c reduction and EPR detection of tempone-H spin trapping. SOD inhibition of the signals in both assays indicates that the signals detected in each assay technique are superoxide mediated. The increased degree of scatter in Panel B relative to Panel A is due to SOD inhibiting tempone production from the variable tempone-H auto-oxidation rates. The comparison of the EPR calibration curve to tempone standards, Figure 5.15.2 panel C, reveals that the linear relationship that exists between the assay methods operates at a 1:1 level – i.e. when the ferricytochrome c assay reports a 1 mole signal, this will be detected as 1 mole of tempone by EPR. This finding agrees with the finding that 1mM tempone-H is sufficient to trap virtually all superoxide in solution at these superoxide production rates (Figure 5.13.5).

5.16: Respiration rates of mitochondrial suspensions in the presence of tempone-H and tempone:

Superoxide production from the mitochondrial Electron Transport Chain is dependent on the respiratory activity. To determine if Tempone-H or Tempone had an effect upon mitochondrial oxygen consumption rates, an investigation of pyruvate malate respiration rates was carried out using a Clark Oxygen Electrode in the presence and absence of tempone-H or tempone, each to a final concentration of 1mM.

The data presented in Table 5.16.1 shows that neither tempone-H nor tempone had any significant effect upon mitochondrial oxygen consumption rates when provided with pyruvate/malate. This indicates that neither tempone-H nor tempone affect the maximal rate of electron transport chain activity (State 3 and uncoupled measures), nor do they affect the permeability of the inner mitochondrial membrane (state 4 measures) at a final concentration of 1mM. This data suggests that tempone-H and tempone do not affect respiratory capacity or coupling, thereby indicating they have potential uses in the measurement of superoxide production by the electron transport chain.
Table 5.16.1: Oxygen consumption rates of mitochondrial preparations prepared from young adult wild-type mice provided with pyruvate/malate in and presence and absence of tempone-H/tempone. Rates are expressed as nmoles/min/mg protein.

<table>
<thead>
<tr>
<th></th>
<th>Control (Mean ± SEM)</th>
<th>n</th>
<th>+ Spin Trap (Mean ± SEM)</th>
<th>n</th>
<th>P-value Student’s t-test</th>
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<tbody>
<tr>
<td><strong>Tempone-H</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>State 3</td>
<td>66.2 ± 8.7</td>
<td>4</td>
<td>70.4 ± 6.2</td>
<td>4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>State 4</td>
<td>16.5 ± 4.2</td>
<td>4</td>
<td>17.3 ± 3.7</td>
<td>4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>FCCP Uncoupled</td>
<td>70.9 ± 7.1</td>
<td>4</td>
<td>67.1 ± 9.2</td>
<td>4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>Tempone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>State 3</td>
<td>71.8 ± 6.3</td>
<td>3</td>
<td>68.4 ± 5.9</td>
<td>3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>State 4</td>
<td>16.2 ± 3.1</td>
<td>3</td>
<td>15.4 ± 2.8</td>
<td>3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>FCCP Uncoupled</td>
<td>75.9 ± 5.8</td>
<td>3</td>
<td>70.7 ± 6.8</td>
<td>3</td>
<td>&gt;0.05</td>
</tr>
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Note that controls and plus spin-trap experiments were performed using the same mitochondrial preparation.

5.17: Identification of a negative going EPR signal using isolated whole mitochondria and tempone-H/CP-H:

Initial experiments were carried out to determine the feasibility of detecting superoxide production by mitochondria using EPR detection of tempone production. As tempone-H auto-oxidizes in solution leading to the formation of a tempone EPR signal (see Appendix Section A3.1), samples containing mitochondrial suspension were run in parallel with control samples lacking mitochondrial suspension. The data in Figure 5.17.1 demonstrates that the addition of whole mitochondrial suspension (to a final concentration of approximately 1mg protein/ml) prepared from wild-type mouse brain resulted in a considerable attenuation of tempone EPR signal development relative to the auto-oxidation control rate. Panel A shows the development of tempone EPR signals over time from auto-oxidation of tempone-H in chelexed buffer both in the presence and absence of mitochondrial suspension, and clearly demonstrates that mitochondria can inhibit the evolution of tempone EPR signals. Panel B shows the magnitude of tempone
signal inhibition normalized to protein concentration, and illustrates the potent antioxidant effect mediated by mitochondrial suspension.

Figure 5.17.1: Effects of whole mitochondrial suspension upon EPR signal evolution. Panel A: Spectra and graph of EPR intensities from a single EPR experiment investigating the effects of mitochondrial suspension upon tempone-H auto-oxidation. EPR spectra were measured at set time intervals in the absence (filled circles, black spectra) and presence (open circles, red spectra) of 0.513mg of mitochondrial protein. Linear regressions applied to all data within no mitochondria (black line) and plus mitochondria (red line) datasets. Panel B: Development of a loss of tempone EPR signal relative to auto-oxidation control expressed per mg of mitochondrial protein. n = 3 from mitochondrial preparations from 3 wild-type OLA129 mice. Error bars represent SEMs.
All repetitions of this experiment demonstrated an approximate 50% inhibition of the auto-oxidation rate of tempone-H in the presence of mitochondria. The variation of the data seen in panel B derived from variability in tempone-H auto-oxidation rate rather than mitochondrial percentage inhibition of the tempone signal (after 150 minutes percentage inhibition 48.34% ± 0.7 SEM, absolute inhibition 2.77 nmoles/mg protein ± 0.36). Overall this result indicates that rather than detecting superoxide production by the mitochondria, incubation of mitochondrial suspension with tempone-H detects an anti-oxidant property of the mitochondria which masked any potential superoxide generation.

To determine if superoxide production could be measured using EPR detection of tempone-H oxidation under conditions where superoxide production from Complex 1 is maximal, tempone signal evolution was followed for mitochondrial samples treated with 8μM rotenone (a rotenone concentration demonstrated to completely inhibit oxygen consumption in a Clark oxygen electrode). A negative going signal relative to the auto-oxidation control rate was again observed (data not shown) indicating that the antioxidant properties of the mitochondrial suspension dominate over any superoxide production using this assay method, and therefore measurement of superoxide production using tempone-H and EPR is not possible in intact mitochondrial preparations. The presence of rotenone in sufficient concentrations to fully inhibit the respiratory chain results in a significantly increased auto-oxidation rate of tempone-H. As the antioxidant properties of mitochondrial suspensions can lessen tempone signal development from auto-oxidation, the effect of rotenone upon the auto-oxidation rate in the presence of mitochondria cannot be determined. Therefore it was not possible to obtain a perfect control sample for EPR measurement of superoxide production from the blocked respiratory chain (i.e. mitochondria in the presence of the inhibitor with no blockage of the respiratory chain).

Irrespective of this, initial experiments were carried out to determine if the addition of rotenone increased superoxide production relative to a control containing mitochondria
but no inhibitor. No significant increase in superoxide production was detected following the addition of rotenone to mitochondrial preparations from wild-type mouse brain (n = 2, data not shown). This suggests that the mitochondrial antioxidant capacity is capable of out-competing the tempone-H for superoxide.

There are a number of potential candidate antioxidants systems which may produce this effect. However interpretation of data is hindered by the various antioxidant systems being able to effect different portions of the tempone produced via auto-oxidation.

\[
\begin{align*}
M^{n+} + O_2 &\rightarrow M^{(n+1)+} + O_2^* \\
M^{(n+1)+} + \text{Tempone-H} &\rightarrow \text{Tempone} + M^{n+} \\
\text{Tempone-H} + O_2^* &\rightarrow \text{Tempone} + H_2O_2
\end{align*}
\]

*Figure 5.17.2: Diagram showing the sources of tempone from auto-oxidation and the antioxidant systems that potentially could reduce tempone concentrations*

A portion of the tempone produced via tempone-H auto-oxidation is superoxide dependent (Figure 5.17.2 right-hand reaction). This portion of tempone production could be inhibited by enzymatic antioxidants catalytically removing superoxide before it can react with tempone-H. The high levels of CuZn and Mn SOD found in mitochondria (in the intermembrane space and matrix respectively) suggests that superoxide removal prior to reaction with tempone-H is feasible. This could potentially
account for the negative tempone signal when mitochondrial suspension was present relative to auto-oxidation control.

Low molecular weight antioxidants such as ascorbate and GSH have been shown to reduce tempone (see Appendix Sections A4.1 and A4.2). Their presence within the mitochondrial suspension could therefore cause the reduction of tempone formed during auto-oxidation via either of the auto-oxidation reactions. This would result in the tempone concentration being lower than in the auto-oxidation control sample, thereby producing the observed result.

The final possibility is that as tempone-H auto-oxidation requires the participation of redox-active metals, the presence of chelating compounds within the mitochondrial suspension may result in a reduced auto-oxidation rate due to decreased metal availability, thereby reducing tempone EPR signals when mitochondrial suspension was present.

Any one or a combination of these antioxidant systems may participate in the production of the negative going EPR signal relative to auto-oxidation control in the presence of mitochondria. As CP-H has been shown to exhibit increased resistance to some low molecular weight antioxidants relative to tempone-H (see Appendix Sections A4.1, A4.2, A5.2 and (Dikalov et al., 1997b)), the possibility of using it as an alternative to tempone-H for detecting superoxide production by mitochondria was investigated.
Figure 5.17.3: Development of a loss 3-CP EPR signal relative to auto-oxidation control and normalized to mitochondrial protein content for wild-type (filled circles) and PrP-null (open circles) mitochondrial preparations. $n = 3$ from 3 separate mitochondrial preparations for each genotype. Error bars represent SEMs.

The data in Figure 5.17.3 demonstrates that the addition of mitochondrial suspension (prepared from either PrP-null or wild-type mouse brain) to a protein concentration of approximately 1 mg/ml results in a significant reduction in the rate of 3-CP EPR signal evolution relative to auto-oxidation control. The magnitude of the inhibition of 3-CP EPR signal evolution following the addition of mitochondrial suspension was routinely around 50% of the auto-oxidation rate, as seen in the case of incubation of mitochondria with tempone-H. In absolute terms, the magnitude of the inhibition of EPR signal development was approximately 3-fold greater when using CP-H rather than tempone-H. This can be explained by the fact that CP-H auto-oxidizes more rapidly than an equimolar solution of tempone-H (see Appendix Section A5.2) thereby leading to a greater signal against which the antioxidant(s) can operate. As the rate of 3-CP EPR signal evolution was attenuated by approximately 50% relative to auto-oxidation controls (i.e. approximately the same percentage inhibition as observed with tempone-H) the loss of 3-CP signal appears to be no less susceptible to antioxidants than the tempone signal if
the increased auto-oxidation rate is taken into account. This indicates that the antioxidant effect is probably not due to the ascorbate or GSH reducing tempone.

Whether the mitochondrial suspension was prepared from wild-type or PrP-null mouse brain did not appear to have a great effect upon the magnitude of the loss of EPR signal when CP-H was incubated with mitochondrial suspension. Assuming the magnitude of the loss of 3-CP EPR signal evolution is proportional to the strength of the antioxidant system, this would suggest that the antioxidant system responsible for the signal inhibition is of approximately equal strength between the genotypes. However as inhibitions of approximately 50% of the auto-oxidation rate were consistently observed irrespective of variations in the protein concentration of the mitochondrial suspension, it is possible that the reaction responsible for the negative going signal was saturated at the concentrations of mitochondria used and hence would be unable to detect any subtle changes between the antioxidant properties of the two genotypes. Therefore no conclusions about the relative potency of the antioxidant system between the genotypes can be made from the results of these experiments.

As the rate of EPR signal evolution due to auto-oxidation was routinely attenuated by around 50% following the addition of mitochondria, it was hypothesised that the antioxidant system was saturated when the spin-trap auto-oxidation rate was inhibited by 50%. To determine if this was the case, various concentrations of mitochondrial suspension from single stocks were incubated with 1mM tempone-H and the rate of tempone EPR signal evolution was measured.
Figure 5.17.4: Loss of Tempone EPR signals following addition of various concentrations of mitochondria suspension. Each point represents a tempone formation rate determined across 3 time-points expressed relative to a control auto-oxidation rate in the absence of mitochondria. Data presented was acquired using 4 separate preparations of PrP-null mitochondria. Curve fitting was carried out using a exponential rise to max single 2 parameter curve fitting algorithm.

The data presented in Figure 5.17.4 demonstrates that the antioxidant effects mediated by mitochondrial suspensions are saturable at a level of approximately 50% loss of the tempone signal evolution rate. The action of low molecular weight antioxidants would not be expected to saturate at a level of 50% inhibition of the auto-oxidation rate as ascorbate and glutathione have been demonstrated to be capable of completely abolishing tempone EPR signals (see Appendix Section A4.1 and A4.2). This result would therefore suggest that the antioxidant properties displayed by mitochondrial suspensions upon tempone-H auto-oxidation are mediated by SOD enzymes as only 50% of tempone-H auto-oxidation is SOD sensitive. This result agrees with the earlier finding that low concentrations of tempone (similar to those reached during tempone-H auto-oxidation within this experiment) are not susceptible to reduction by reduced
glutathione (data not shown), which suggested that glutathione is incapable of producing the observed tempone signal inhibition.

EPR signal inhibition was found to be greater in samples where mitochondria were incubated with CP-H (Figure 5.17.3) than samples where mitochondria were incubated with tempone-H (Figure 5.17.1). This can be explained by the fact that the approximate 50% inhibition of hydroxylamine spin-trap auto-oxidation will be greater in samples containing CP-H due to the increased propensity of CP-H to auto-oxidize relative to tempone-H (see Appendix Section A5.2).

5.18: Studies of the antioxidant capacity of submitochondrial particles using electron paramagnetic resonance spectroscopy and a spectrophotometric superoxide dismutase assay:

As measurement of superoxide production by mitochondria was hindered by the activity of antioxidants, the use of SubMitochondrial Particles (SMPs) within EPR experiments was considered as an alternative system in which to measure superoxide production by the respiratory chain. In order to determine whether SMPs produced using the procedure outlined in Section 4.9 possessed the antioxidant defences present in mitochondrial preparations, a SOD assay and a tempone reduction assay were performed using SMPs.

The data in Figure 5.18.1 demonstrates that the SOD and tempone reducing antioxidant properties of isolated mitochondria (see Table 4.16.1 and Figure 5.20.1 respectively) are not observable in either wild-type or PrP-null SMP preparations. The most likely explanation for the loss of this antioxidant effect is that the responsible compound(s) have been released from the mitochondria during mitochondrial lysis steps, and removed in the supernatant fractions during the submitochondrial particle wash steps. The data also confirms that the antioxidant effect is not a property of the inner mitochondrial membrane, due to the loss of antioxidant effect whilst SMP respiratory capacity is maintained.
Figure 5.18.1: Antioxidant Properties of SMPs. Panel A: Wild type (black bar) and PrP-null (grey bar) SMP SOD activity determined by percentage inhibition of NBT reduction. $n = 8$ for all bars, error bars represent SEMs. Statistics were performed as Student’s unpaired t-test, no significant difference between the genotypes was detected.

Panel B: Effects of wild-type (black bar) and PrP-null (grey bar) SMP preparations upon tempone EPR signals relative to tempone standard control (white bar) following incubation at 37°C for 2 hours. $n = 3$, error bars represent SEMs.

The lack of a tempone EPR signal reduction (Figure 5.18.1 panel A), and the lack of SOD activity (Figure 5.18.1 panel B) in the SMP preparation permitted an assay of superoxide generation from the respiratory chain without the need to consider antioxidant defences as a possible source of variation between the genotypes.

It is important to note that the protein concentrations of SMPs used during these experiments were selected to mimic the concentrations of mitochondria used in the assessment of mitochondrial antioxidant activity. As SMP preparation involves purification of mitochondrial membranes relative to isolated mitochondrial preparations, the SMP preparations are enriched with mitochondrial membrane and therefore the lack of antioxidant effect is unlikely due to the use of insufficient SMP suspension.
5.19: Assay superoxide production by the respiratory chain in submitochondrial particles using electron paramagnetic resonance spectroscopy and tempone-H:
The addition of SMP preparations to tempone-H solutions had no discernible effect on the rate of tempone signal evolution relative to auto-oxidation control samples (data not shown). This result agrees with the previous findings that SMP preparations have no direct antioxidant effect upon tempone EPR signals, nor possess any SOD activity. This data also indicates that the SMP preparation does not possess a chelating compound capable of reducing tempone-H auto-oxidation via removal of free metal ions from solution. No superoxide production was detectable from the SMPs following addition of substrate alone, which is unsurprising considering the SMP preparations used were shown to be uncoupled in a Clark Oxygen Electrode (see Section 4.22). However when substrate was provided and the respiratory chain inhibited at an appropriate downstream point, superoxide production by the respiratory chain could be measured as a positive EPR signal relative to control. A thorough analysis of superoxide production by the respiratory chain of SMP prepared from wild-type and PrP-null mouse brain was therefore carried out. All SMP suspensions utilized for EPR experiments demonstrated oxygen consumption when provided with NADH or succinate but not when provided with pyruvate and malate when tested in a Clark Oxygen Electrode. Complete inhibition of oxygen consumption was confirmed in the oxygen electrode for each inhibitor concentration used in every preparation.

To correct for auto-oxidation of tempone-H the superoxide generation rates of all samples are expressed relative to a control sample which lacked any downstream inhibitor. The effects of all inhibitor concentrations used upon tempone-H auto-oxidation rates were determined, and where necessary additional control samples were run to allow correction for these increased auto-oxidation rates. This was particularly important in the case of KCN-mediated inhibition of the respiratory chain due to both its considerable effect upon auto-oxidation rates, and the inability to verify signals as being superoxide dependent due to KCN being an inhibitor of CuZn SOD.
Figure 5.19.1: Spectra for a single SMP superoxide production experiment with data waveform subtracted for the correct auto-oxidation control for each spectra. Data was acquired following incubation of samples for 30 minutes at 30°C.

The results of the EPR study of superoxide production by the respiratory chain in SMPs are displayed in Table 5.19.1. For details of the points of entry of substrates and points of action of inhibitors refer to Figure 4.2.2. Following the addition of the Complex I substrate NADH and the Complex I blocker rotenone to SMP preparations a clearly observable rate of tempone production occurred. This signal was verified as superoxide dependent by its inhibition by SOD. Following the addition of KCN to SMPs provided with NADH as respiratory substrate, a rate of superoxide production lower than that observed in the presence of rotenone was detected. The rates of superoxide production by SMPs provided with NADH as respiratory substrate and blocked at either Complex I or IV were statistically significantly different between wild-type and PrP-null SMP preparations. PrP-null SMP suspensions generated approximately 0.08 nmoles per
min/mg protein (approximately 50%) more superoxide than wild-type controls. This increased rate of superoxide radical production in SMP suspensions from PrP-null mice could be attributed to Complex I as it was detectable when a Complex I substrate was provided during blockade of Complex I by rotenone. It should also be noted that this is the respiratory Complex which was observed to have augmented function during the oxygen electrode studies of mitochondrial and SMP respiration (see Tables 4.19.1, 4.20.1 and 4.22.1).

Table 5.19.1: Rates of superoxide production (nmoles/min/mg protein) of wild type and PrP-Null SMPs determined by EPR detection of tempone-H oxidation.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type (Mean ± SEM)</th>
<th>PrP-null (Mean ± SEM)</th>
<th>n</th>
<th>P-value 2-Way ANOVA Tukey Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Rotenone</td>
<td>0.151 ± 0.021</td>
<td>0.229 ± 0.024</td>
<td>9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>+Rotenone &amp; SOD</td>
<td>0.014 ± 0.017</td>
<td>0.012 ± 0.015</td>
<td>9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>+KCN</td>
<td>0.117 ± 0.018</td>
<td>0.199 ± 0.029</td>
<td>9</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

| Succinate      |                        |                       |    |                               |
| +Myxothiazol   | 0.009 ± 0.014          | 0.020 ± 0.011         | 9  | >0.05                         |
| +Myxothiazol & SOD | 0.010 ± 0.012   | 0.012 ± 0.015         | 9  | >0.05                         |
| +Antimycin A   | 0.119 ± 0.010          | 0.117 ± 0.006         | 9  | >0.05                         |
| +Antimycin A & SOD | 0.037 ± 0.010   | 0.029 ± 0.018         | 9  | >0.05                         |
| +KCN           | 0.027 ± 0.009          | 0.029 ± 0.013         | 9  | >0.05                         |

n numbers represent experimental runs, each from a separate animal

Statistics were performed as 2-Way ANOVAs across blockade points within each substrate.

When SMPs were provided with the Complex II substrate succinate, EPR measurements were performed when Complex I was inhibited using rotenone to eliminate any possibility of reverse electron flow which could potentially generate considerable
quantities of superoxide. Superoxide production rates were low when the electron transport chain provided with succinate as respiratory substrate was inhibited by either myxothiazol or KCN. In contrast a considerable rate of superoxide production was detectable following the addition of the Complex III inhibitor antimycin A. The magnitude of this superoxide production was similar in both genotypes, indicating that superoxide production under antimycin A blockade of Complex III is unchanged between the genotypes. The fact that a difference in superoxide production was observed from Complex I but not from Complex III in the same SMP preparations provides evidence that the differences detected were not an artefact due to SMP sample purity.

![Figure 5.19.2: Contribution of peroxynitrite towards tempone EPR signals from SMPs. Tempone-H oxidation mediated by Complex I of the respiratory chain was determined by EPR following rotenone blockade of Complex I in SMPs suspensions from Wild-type (black bars) and PrP-null (grey bars) animals. Control sample contains SMP suspension, rotenone and tempone-H. All spectra were background subtracted to compensate for tempone-H auto-oxidation using a sample lacking rotenone. Superoxide dependence of the signal was verified by inhibition of the signal by dialysed CuZn SOD to a concentration of 20U/ml, and lack of signal inhibition following addition of the competitive NOS inhibitor L-NAME to a concentration of 1mM. n = 2. Error bars represent the spread of the data.](image-url)
As tempone-H is thought to be capable of spin trapping peroxynitrite as well as superoxide (Dikalov et al., 1997a), it was necessary to verify whether peroxynitrite contributed towards the EPR signals detected from SMPs. The data in Figure 5.19.2 demonstrates that on addition of rotenone to SMP suspensions a tempone EPR signal was detectable relative to auto-oxidation control as previously reported. This signal was inhibited by the addition of SOD and unaffected by the addition of the competitive NOS inhibitor L-NAME. To ensure that L-NAME did not significantly alter tempone-H auto-oxidation rates via introduction of redox-active metals, a control in the absence of rotenone was carried out and revealed that addition of L-NAME to a final concentration of 1mM does not significantly elevate tempone-H auto-oxidation rates. These results suggest that tempone-H oxidation by peroxynitrite does not significantly contribute towards the tempone EPR signals detected following blockade of the respiratory chain of SMPs, thereby indicating that the signals were superoxide dependent. This finding is of importance as it indicates that the increased Complex I mediated superoxide production in SMP suspensions from PrP-null animals relative to wild-type controls is not due to altered nitric oxide production or peroxynitrite formation.

5.20: Studies of mitochondrial antioxidant capacity using electron paramagnetic resonance spectroscopy detection of tempone reduction:

The preceding data clearly demonstrates that mitochondrial suspensions have a potent antioxidant effect upon the auto-oxidation of the hydroxylamine spin traps tempone-H and CP-H. This effect appears to be mediated by SOD enzymes contained within the mitochondria as the effect is saturable at a level of approximately a 50% reduction in the tempone-H auto-oxidation rate. However further experiments were carried out to assess whether mitochondrial suspensions possessed low molecular weight antioxidants capable of reducing tempone. This was achieved by incubating mitochondrial suspensions in the presence of 500μM tempone and monitoring the resulting EPR spectrum over time.
This tempone reduction assay for measuring mitochondrial antioxidant capacity provides
a measure of only antioxidants capable of reducing tempone, rather than a combined
measure of the effects of SOD, metal chelation and tempone-reducing antioxidant
activity. Tempone rather than 3-CP was utilized for these experiments due to its greater
susceptibility to reduction by antioxidants.

Figure 5.20.1: The effects of the quantity of mitochondrial protein present upon the loss
of tempone EPR signals for wild-type (filled circles) and PrP-null (open circles)
following 2 hours incubation at 37 °C. Data acquired from 3 wild-type and 2 PrP-null
mitochondrial preparations. Linear regressions were applied to all data within wild-
type (blue line) and PrP-null (red line) genotype datasets. Spectra shown were acquired
for the PrP-null samples labelled *, and are shown following waveform subtraction from
the tempone control sample. Note that waveforms shown are negative due to the loss of
tempone signal in the presence of mitochondrial suspension.

To determine if the magnitude of the loss tempone EPR signal was proportional to the
quantity of antioxidants present, the correlation between the amount of mitochondrial
protein present and the magnitude of EPR signal loss was determined. The data in
Figure 5.20.1 demonstrate that the addition of mitochondrial suspensions result in a loss
of tempone EPR signals in a concentration dependent manner which varied linearly with mitochondrial protein concentration within the ranges tested. When the relationship between protein content and EPR signal loss was plotted for data acquired for 3 and 4 hours of incubation, the linear relationship was maintained (data not shown). These findings suggest that data may be normalization to protein concentration offline.

![Bar chart showing the ability of mitochondrial suspensions, prepared from wild-type (black bars) and PrP-null (grey bars) mice, to reduce tempone EPR signals, expressed as nmoles of tempone reduced per mg of mitochondrial protein.](image)

**Figure 5.20.2:** Bar chart showing the ability of mitochondrial suspensions, prepared from wild-type (black bars) and PrP-null (grey bars) mice, to reduce tempone EPR signals, expressed as nmoles of tempone reduced per mg of mitochondrial protein. *n* = 10 for all bars except PrP-null four hour point for which *n* = 9. Statistics were performed as 1-Way ANOVAs between the genotypes, no significant differences were detected for any of the time points. Error bars represent SEMs.

The data in Figure 5.20.2 shows that there is no significant difference between the abilities of wild-type and PrP-null to reduce tempone to an EPR silent species. This indicates that the net effects of the species responsible for the inhibition of tempone EPR signals are similar between the genotypes.
In an attempt to identify the species responsible for the tempone EPR signal inhibition, the possibility of preventing the mitochondrial effect on tempone EPR signals was investigated using hydrogen peroxide and CDNB.

To attempt to remove the ability of mitochondrial suspensions to reduce tempone, aliquots of mitochondrial suspensions were pre-treated with hydrogen peroxide which was washed-out prior to the addition of tempone. This protocol attenuated the ability of mitochondria to reduce tempone EPR signals. To determine a suitable concentration of hydrogen peroxide to use within these experiments to produce a maximal effect, a concentration effect relationship was determined.

![Figure 5.20.3: The effects of pre-treatment of mitochondria with varying concentrations of hydrogen peroxide upon the ability of wild-type (filled circles) and PrP-null (open circles) mitochondrial suspensions to inhibit tempone EPR signals following 2 hours incubation at 37°C. n = 3 for all points. Error bars represent SEMs](image)

The data in Figure 5.20.3 demonstrates that the hydrogen peroxide treatment protocol employing the wash step produced a considerable inhibition of their ability to inhibit tempone EPR signals. To ensure maximal inhibition of the antioxidant effect, further experiments employed a final concentration of 0.9% hydrogen peroxide.
An experiment was carried out to determine if the effects of hydrogen peroxide upon the mitochondrial antioxidants responsible for tempone reduction were similar between the genotypes.

![Graph showing the effects of hydrogen peroxide treatment upon wild-type (black) and PrP-null (grey) mitochondrial suspension mediated loss of tempone EPR signals.](image)

**Figure 5.20.4:** The effects of hydrogen peroxide treatment upon wild-type (black) and PrP-null (grey) mitochondrial suspension mediated loss of tempone EPR signals. \( n = 12 \), triplicate readings taken from each mitochondrial preparation. EPR readings were taken following 2 hours incubation at 37°C. Error bars represent SEMs.

The data in Figure 5.20.4 shows that following hydrogen peroxide pre-treatment, mitochondrial suspensions have a lowered ability to reduce tempone EPR signals by approximately 75% in both genotypes. This indicates that the effect of the reducing agent responsible can be significantly attenuated by the application of hydrogen peroxide.

To determine if the mitochondrial antioxidant effect was due to the activity of reduced glutathione which is known to be able to reduce tempone EPR signals (see Appendix Section A4.2), the ability of mitochondrial suspensions to lower tempone EPR signals was measured following pre-treatment with 1-chloro-2,4-dinitrobenzene (CDNB), which conjugates glutathione by the action of Glutathione-S-Transferase enzymes (Habig *et al.*, 1974). To determine the appropriate concentration of CDNB to use to produce a
maximal reduction in tempone EPR signal loss, a concentration effect relationship was determined.

Figure 5.20.5: The effects of CDNB concentrations upon wild-type (filled circles) and PrP-null (open circles) mitochondrially-mediated loss of tempone EPR signals. n = 3 for all points, error bars represent SEMs

The data in Figure 5.20.5 demonstrates that the treatment of mitochondria with CDNB resulted in a diminished ability to reduce tempone EPR signals to a maximum level of approximately 15nmol/mg protein. The maximal effect was reached when CDNB concentrations of 0.5mM or higher were used. The effects of CDNB on tempone signal inhibition were similar between genotypes, indicating that CDNB has a similar potency within the mitochondrial preparations from the two genotypes used. This allows the use of a single concentration of CDNB to maximally dis inhibit tempone signals in wild-type and PrP-null mitochondrial preparations.

The magnitudes of the reductions in tempone EPR signal loss were compared between the genotypes using 1mM CDNB to ensure that CDNB was maximally effective.
The data in Figure 5.20.6 shows that treatment of mitochondria with 1.0mM produced an approximate 20% decrease in the ability of mitochondrial preparations to reduce tempone EPR signals. The magnitude of the effect observed was similar between the genotypes, indicating that the portion of the tempone signal loss mediated by GSH is similar between the genotypes.

To determine if the reductions of tempone EPR signal loss by CDNB and \( \text{H}_2\text{O}_2 \) occurred due to their actions on the same mitochondrial antioxidant, experiments were carried out to determine if the effects were additive. Addition of \( \text{H}_2\text{O}_2 \) and CDNB individually produced a clear reduction of tempone EPR signal loss as previously reported, however simultaneous addition of \( \text{H}_2\text{O}_2 \) and CDNB did not produce an additive effect, indicating that CDNB disinhibition of tempone EPR signals occurred due to removal of a subset of the antioxidants removed by hydrogen peroxide (data not shown, \( n = 2 \)).
To determine whether cytochrome c, an essential component of the electron transport chain, could be implicated in the inhibition of tempone EPR signals by mitochondria, 500μM tempone was incubated for a period of 2 hours with ferricytochrome c to a concentration of 1mM. Following incubation, tempone EPR signals were unaffected by the presence of ferricytochrome c indicating that it is incapable of inhibiting tempone EPR signals.

The data presented show that mitochondrial suspensions can potently inhibit tempone EPR signals. Whilst the inhibition can be attenuated by around 80% by hydrogen peroxide, and by around 20% by CDNB, it was not possible to conclusively identify the source of the tempone signal inhibition, nor was it possible to completely prevent the EPR signal loss.
Discussion:
5.21: Summary of results of EPR calibration and EPR investigation of mitochondrial superoxide generation:

Complex I of the electron transport chain is widely believed to be a major physiological source of superoxide radicals. As results presented in this thesis indicate increased Complex I activity, and other reports have suggested increased levels of oxidative stress in PrP-null mice, it was of interest to determine whether the respiratory chain produced more superoxide in PrP-null mouse brain. Therefore experiments were carried out to develop a technique permitting quantitative assessment of superoxide production by the respiratory chain.

The hydroxylamine spin-trap tempone-H was characterized with a view to using it to quantitatively assess superoxide production by the mitochondrial respiratory chain. Tempone, formed from oxidation of tempone-H, was found to provide an EPR signal proportional to its concentration and proportional to the concentration of superoxide radical present.

To ensure that tempone-H or tempone did not directly interact with any of the components of the respiratory chain, mitochondrial respiration rates were measured in the presence of 1 mM tempone or 1 mM tempone-H. As mitochondrial oxygen consumption rates were unchanged in the presence of tempone/tempone-H, it can be assumed that neither the spin-trap nor the spin-trap adduct are capable of directly interacting with the respiratory chain and thereby altering the rate of electron flow through the respiratory complexes. This is of particular importance when considering that the rate of electron flow to a specific superoxide generating charge carrier within a respiratory complex may have been disrupted if tempone or tempone-H could interact with the respiratory chain.

However tempone-H is subject to auto-oxidation in solution leading to the production of a background tempone EPR signal in the absence of superoxide. Upon incubating
mitochondria with tempone-H, it was found that an EPR signal smaller than the appropriate auto-oxidation control was generated, presumably due to the antioxidant properties of the mitochondrial suspensions. In order to circumvent the antioxidant effects observed in mitochondrial suspensions, the feasibility of using SMP suspensions to study superoxide production by the ETC was investigated. Submitochondrial particles were considered devoid of SOD activity due their failure to inhibit NBT reduction determined spectrophotometrically, and the lack of any effect on tempone-H auto-oxidation upon addition of SMP suspension. Indeed, if Mn SOD had not been washed out during SMP preparation, higher levels of SOD activity would have been expected in the SMP fraction due to enrichment with mitochondrial components relative to crude homogenate. SMP suspensions were also shown to be unable to reduce tempone, indicating that the antioxidant(s) in mitochondrial suspensions responsible for tempone reduction were also removed during SMP preparation. This suggests that SMPs may be used as a system in which to assess superoxide production by the respiratory chain in the absence of interference by antioxidants.

Oxygen electrode analysis of SMP respiration rates revealed that SMPs were uncoupled but capable of respiration when provided with either Complex I or Complex II substrates. Unsurprisingly tempone-H oxidation by superoxide production from the electron transport chain of uncoupled SMP suspensions was not observed prior to the addition of a respiratory chain inhibitor. However tempone-H oxidation was observed following addition of rotenone or KCN to SMP suspensions provided with the Complex I substrate NADH, and following the addition of antimycin A (but not myxothiazol or KCN) to SMP suspensions provided with the Complex II substrate succinate. The effects of SOD and L-NAME upon tempone-H oxidation by the respiratory chain were assessed to determine whether tempone-H oxidation was superoxide-mediated. Inhibition of the signals by SOD, but not by L-NAME, suggest that the signals detected were superoxide-dependent.
Comparison of rates of superoxide production by SMPs prepared from PrP-null and wild-type mice revealed a statistically significant increase in superoxide production by PrP-null SMPs when provided with Complex I substrates and inhibited using rotenone or KCN, but not when provided with Complex II substrate and inhibited by antimycin A, myxothiazol or KCN.

5.22: Qualitative assessment of respiratory chain superoxide production:
Whilst the reactive oxygen species generated by the respiratory chain is generally accepted to be superoxide, techniques used to assay the production of ROS by the respiratory chain either directly assay superoxide or assay hydrogen peroxide which is formed from dismutation of superoxide. Considerable evidence suggests that two respiratory Complexes are involved in the production of superoxide radicals by the respiratory chain, these are Complex I (Turrens & Boeris, 1980; Turrens et al., 1982; Kushnareva et al., 2002) and Complex III (Boveris et al., 1976; Cadenas et al., 1977; Han et al., 2001). Whilst the exact sites within these Complexes responsible for superoxide generation may remain contentious, the effects of a number of mitochondrial inhibitors upon superoxide production have been characterized and generally accepted.

Qualitatively the results presented in this thesis regarding the points of superoxide production by the respiratory chain agree with previous findings. These include superoxide production when Complex I substrates are provided and the respiratory chain is inhibited by rotenone or KCN at Complex I or IV respectively (Turrens & Boeris, 1980), superoxide production following provision of either Complex I or Complex II substrates and inhibition of the respiratory chain at Complex III by antimycin A (Boveris et al., 1976; Turrens & Boeris, 1980) and that myxothiazol inhibition of Complex III does not result in the production of superoxide when succinate is provided as respiratory substrate and reverse electron transport is inhibited by rotenone (Turrens et al., 1985). The variable effects of antimycin A, myxothiazol and KCN upon rates of superoxide generation by Complex III can be explained by their effects upon the Q-cycle. Antimycin A blocks at a point which stabilizes the ubisemiquinone responsible for
superoxide generation, thereby increasing superoxide production. In comparison
myxothiazol and KCN prevent formation of ubisemiquinone and therefore attenuate
superoxide production by Complex III (Nicholls & Ferguson, 2002).

5.23: Quantitative aspects of superoxide production by the respiratory chain:
Reported rates of superoxide/hydrogen peroxide production by the respiratory chain
vary considerably between studies. This thesis has reported superoxide production rates
of 0.151 and 0.117nmoles/min/mg when SMPs from wild-type mice provided with
Complex I substrates were inhibited with rotenone and KCN respectively, and SMPs
provided with Complex II substrate produced negligible quantities of superoxide under
myxothiazol inhibition and 0.119nmoles/min/mg under antimycin A inhibition of
Complex III. When compared to other studies of superoxide/hydrogen peroxide
production by the respiratory chain these values appear rather low.

Votyakova & Reynolds reported a hydrogen peroxide production rate from rat brain
mitochondria of 0.434nmoles/min/mg when provided with Complex I substrates and
rotenone was present, and 0.275nmoles/min/mg when Complex II substrate was
provided and antimycin A present (Votyakova & Reynolds, 2001). St-Pierre et al
documented hydrogen peroxide production rates of ~0.1, 0.2 and 0.25nmoles/min/mg
from rat liver, skeletal muscle and heart mitochondria respectively when Complex I
substrates are provided and the rotenone present (St-Pierre et al., 2002). Han et al have
reported hydrogen peroxide production rates by rat heart mitochondria of
0.15nmoles/min/mg when Complex I substrates are provided and rotenone present (Han
et al., 2003b) and 0.08nmoles/min/mg when Complex II substrates were provided and
antimycin A present (Han et al., 2003a). Kudin et al have reported a hydrogen peroxide
production rate of 0.68nmoles/min/mg by rat heart mitochondria when Complex I
substrates are provided and rotenone is present, and 0.14nmoles/min/mg when Complex
II substrate is provided and antimycin A is present (Kudin et al., 2004). Vinogradov and
Grivennikova have reported a maximal superoxide production rate of around
1.5 nmoles/min/mg by bovine heart submitochondrial particles provided with Complex I substrates in the presence of rotenone (Vinogradov & Grivennikova, 2005).

A number of factors could underlie the variability in hydrogen peroxide/superoxide production by the respiratory chain detected in these various studies – these include the use of intact mitochondria versus submitochondrial particles, the purity of mitochondrial or submitochondrial particle suspensions, the use of tissue isolated from different species, the use of different body tissues and the use of different substrate/inhibitor concentrations.

A number of possible explanations for the lower rates of superoxide production detected within this study can be put forward.

The SMP suspensions used within this study may be of a lower purity than the preparations used by other groups, thereby reducing the reported superoxide production rates following normalization to protein content. However similarities in the respiration rates of the SMPs used in this study to those reported elsewhere tends to suggest this was not the case.

Cytochrome c may potentially act as an antioxidant, and therefore may compete with tempone-H for superoxide. Such an activity of cytochrome c may be expected to also inhibit the superoxide-dependent portion of tempone-H auto-oxidation – an effect that was not observed. However the production of superoxide by SMPs in relatively close proximity to cytochrome c may result in cytochrome c removing a greater proportion of superoxide produced by the respiratory chain when compared to superoxide produced during auto-oxidation. Whilst such a phenomenon could result in underestimation of superoxide production rates, an alteration in cytochrome c levels between the genotypes would be required to produce the observed differences in superoxide production rates and appears unlikely to be capable of producing a Complex I specific difference.
ImM tempone-H was demonstrated to be capable of reacting with concentrations of superoxide similar to those produced by SMPs when superoxide was produced by a xanthine/xanthine oxidase system. However superoxide production by the enzymatic couple is dispersed throughout the solution whilst SMP superoxide production may be highly localized, resulting in greater heterogeneity of superoxide concentrations. As spontaneous superoxide dismutation is second order with respect to superoxide concentration whilst the reaction of superoxide with tempone-H is first order with respect to superoxide concentration, 1mM tempone-H may react with only a finite proportion of the superoxide produced by the respiratory chain if superoxide concentrations rise in discrete regions. This would lead to an underestimation of all superoxide production rates. However if indeed this is the case, the difference between the PrP-null and wild-type superoxide production rates reported may be a considerable underestimation of the difference between the genotypes.

The experiments of Vinogradov and Grivennikova suggest that superoxide production by Complex I varies with NADH concentration. The relationship is bell-shaped with peak superoxide production rates at around a concentration of 50μM NADH (Vinogradov & Grivennikova, 2005; Grivennikova & Vinogradov, 2006). This is vastly less than the 50mM NADH concentrations used within our experiments and may explain the relatively low rates of superoxide production observed within this study. However in order for this effect to have produced the observed differences between the genotypes the effect of NADH concentration upon superoxide production would have to vary between the genotypes.

Alternatively interaction of the detection system with the respiratory chain could result in over or underestimation of the rate of production of reactive oxygen species by the respiratory chain. This could either be an underestimation of superoxide production in this study, or an overestimation of superoxide production in other studies.
Whilst these mechanisms all provide plausible ways in which the superoxide production rates reported in this thesis may underestimate actual superoxide production rates, none of the mechanisms provide a way in which the difference between the genotypes could be artificial.

5.24: Identification of the point of change in the respiratory chain of PrP-null mice: Irrespective of the quantity of superoxide detected in the current study, a difference in the rate of superoxide production between PrP-null and wild-type controls was detected. An increased rate of superoxide radical generation was detected in SMP suspensions from PrP-null animals relative to wild-type controls when Complex I substrates were provided and the respiratory chain inhibited by rotenone at Complex I. This increased rate of radical formation can be assigned to Complex I as under these conditions electron flow occurs solely through Complex I.

A significant difference in the rates of superoxide production between the genotypes was also identified when SMP suspensions were provided with the Complex I substrate NADH and blocked at Complex IV using KCN. As the magnitude of the difference between the genotypes under these conditions was similar to that observed upon provision of Complex I substrates and inhibition of the respiratory chain at Complex I, it can be assumed that the increased superoxide production of PrP-null under KCN inhibition is also due to increased superoxide production from Complex I. However rates of superoxide production were found to be lower in both genotypes when the respiratory chain was inhibited using KCN rather than with rotenone. Although the concentration of inhibitors used were verified as being capable of completely preventing oxygen consumption, it is possible that the difference in superoxide production is due to partial inhibition of the respiratory chain by KCN.

Superoxide production rates of SMPs provided with succinate as substrate and inhibited using antimycin A showed no difference between the genotypes. As superoxide production rates by Complexes I and III do not change in parallel between PrP-null and
wild-type preparations, the observed difference is not due to an alteration in sample yield or purity between the genotypes, and helps confirm the existence of a true difference in Complex I superoxide production rates between the genotypes.

This assignment of increased superoxide production in PrP-null SMP preparations to Complex I is in agreement with previous findings which indicate increased transcription of a Complex I subunit (Miele, 2000) and the Complex I specific increase in maximal oxygen consumption reported in this thesis.

5.25: Assignment of the increased rate of tempone-H oxidation in PrP-null SMP suspensions to increased superoxide radical production by Complex I:

Experimental observations have suggested that in addition to trapping superoxide radicals, tempone-H may also be oxidized to produce tempone by peroxynitrite anions. Peroxynitrite generated by SIN-1 (which produces superoxide and nitric oxide which react at diffusion controlled rates producing peroxynitrite) reacts with tempone-H to produce a tempone spin signal (Dikalov et al., 1997a), and such signals are unlikely to be due to the direct oxidation of tempone-H by superoxide as SIN-1 fails to produce a signal using a nitric oxide specific electrode (Taylor et al., 2004).

As tempone-H can react with peroxynitrite to produce a tempone spin signal, it is necessary to consider whether the signals reported here could, at least in part, be produced by peroxynitrite oxidation of tempone-H. The rate constants for the reactions of tempone-H with peroxynitrite and superoxide have been reported as $6 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ and $1.2 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ respectively (Dikalov et al., 1997a), whilst the reaction of nitric oxide with superoxide is essentially diffusion controlled with a rate constant around $6.7 \times 10^9 \text{M}^{-1}\text{s}^{-1}$ (Huie & Padmaja, 1993). Whilst 100% of the superoxide produced by a simple enzymatic couple has been determined to react with tempone-H, it remains uncertain whether all peroxynitrite would be trapped by tempone-H due to its greater reactivity with cellular macromolecules. Nitric oxide production by mitochondria leading to peroxynitrite formation may therefore result in only partial trapping of the
superoxide radicals produced. Furthermore the reaction of peroxynitrite with tempone-H occurs via a two-step process which oxidizes two moles of tempone-H for every mole of peroxynitrite (Dikalov et al., 1997a). Altered peroxynitrite formation could therefore be envisaged to either increase tempone production (via altered stoichiometry of reaction) or decrease tempone production (via partial spin trapping).

Studies of nitric oxide synthase activity in PrP-null mice have produced conflicting results. Zurich PrP-null mice have been reported to display decreased neuronal nitric oxide synthase (nNOS) activity and a delocalization of nNOS from lipid rafts (Keshet et al., 1999). In comparison Wong et al uncovered an increase in nNOS expression in a doppel overexpressing PrP-null mouse, but not in the Edinburgh PrP-null mouse (Wong et al., 2001c), although NOS activity was not assessed. Whilst altered nitric oxide production could affect tempone-H oxidation via variable peroxynitrite formation, the results of Wong et al, carried out using the same strain of PrP-null mouse as utilized for the experiments reported in this thesis, suggest that it is unlikely that nitric oxide synthase activity is altered under our experimental conditions.

In addition a number of factors suggest that nitric oxide production will be minimal under the conditions used within this study to study superoxide production rates. The SMP suspensions had their matrix contents dialysed out thereby removing the nitric oxide synthase (NOS) substrate arginine, and calcium ions which are necessary for NOS activity are maintained at low concentrations due to the presence of EDTA. It has also recently been argued that mitochondria in mouse brain contain minimal concentrations of NOS (Lacza et al., 2004), which would further exclude the possibility that peroxynitrite generation contributes to the tempone EPR signal seen within this study.

Whilst considerable evidence suggests that the differences in tempone-H oxidation rates between the genotypes are not due to altered NOS activity, a number of control experiments were carried out to conclusively eliminate peroxynitrite formation as a potential source of variation between the genotypes. The use of SOD enzymes to
catalytically remove superoxide radicals provides one avenue via which EPR signals may be demonstrated to be superoxide-dependent. Whilst 20U/ml CuZn SOD is capable of removing superoxide prior to its reaction with tempone-H, 20U/ml CuZn SOD has been suggested to be unable to out-compete nitric oxide for superoxide (Taylor et al., 2004). The almost complete abolition of tempone EPR signals using 20U/ml SOD suggests that the signals were not peroxynitrite dependent. Secondly the addition of a non-selective competitive NOS inhibitor had no effect upon the rates of superoxide generation following rotenone blockade in either PrP-null or wild-type samples. This indicates that tempone-H oxidation was not dependent upon nitric oxide production. And finally alteration in nitric oxide production, and therefore peroxynitrite formation, may be expected to alter the rate of tempone-H oxidation from all points of the respiratory chain, whilst only a Complex I specific effect was observed within the current study.

Therefore the methodology employed and the use of appropriate control experiments within the current study permits elimination of the possibility that the observed differences between the genotypes were due to altered NOS activity, and therefore the differences represent altered superoxide production rates.

5.26: Modulators of Complex I superoxide production:
The detection of a Complex I specific increase in superoxide production by PrP-null SMP suspensions indicates that some alteration in Complex I is responsible for the difference. A number of reports in the literature suggest a variety of factors can modulate superoxide production from respiratory Complexes independent of the expression of Complex I. Such a phenomenon could account for the difference in superoxide production rates observed between the genotypes.

The rate of superoxide production by the respiratory chain has been shown to be related to the redox state of NAD(P)H and the mitochondrial membrane potential in isolated mitochondrial preparations (Starkov & Fiskum, 2003). Increasing the mitochondrial
membrane potential or shifting the redox state of NAD(P)H towards a greater state of reduction leads to increased superoxide production from the respiratory chain (Starkov & Fiskum, 2003). However two factors suggest that such a mechanism does not underlie the difference in superoxide generation rates between the genotypes reported in this thesis. Firstly our observation of increased superoxide production in PrP-null mouse mitochondria was obtained under rotenone (or KCN) inhibition of the respiratory chain, meaning that the mitochondrial membrane potential will not influence the rate of superoxide production. And secondly our measurements were performed in uncoupled SMPs suspensions which indicates that $V_m$ will be significantly lowered. The observation that the mitochondrial membrane potential in PrP-null mouse brain is unchanged relative to wild-type controls (Lobao-Soares et al., 2005) also suggests that an alteration in mitochondrial membrane potential is unlikely to be a factor altering superoxide production from the respiratory chain in uninhibited mitochondria from PrP-null mice.

Glutathionylation of Complex I during oxidative stress results in a decrease in respiratory activity and an increase in superoxide radical production by the Complex (Beer et al., 2004). Increased levels of markers of oxidative stress observed in PrP-null mice (Wong et al., 2001b) suggests that glutathionylation of Complex I may be increased in PrP-null mice, thereby increasing superoxide radical production from the Complex under physiological conditions. However this effect might not persist following washout of the mitochondrial matrix during SMP preparation and, as this scheme relies upon inhibition of Complex I to increase superoxide production, altered glutathionylation does not provide an explanation for the observed increase in superoxide radical production observed in PrP-null mice when Complex I is inhibited with rotenone. Increases in calcium concentrations have also been determined to inhibit Complex I activity in rat heart mitochondria in a superoxide dependent process (Sadek et al., 2004). Whilst this mechanism provides a scheme via which Complex I activity and superoxide levels are closely linked, it would not provide a mechanism by which to increase Complex I activity in a state of oxidative stress.
Similarly superoxide production by Complex I has been demonstrated to be increased following incubation of mitochondrial preparations with nitric oxide (Riobo et al., 2001). Alteration in nNOS activity has been demonstrated in the Zurich PrP-null mouse (Keshet et al., 1999), although a study of the Edinburgh PrP-null mouse detected no difference in nNOS or iNOS expression (Wong et al., 2001c). However even if NOS activity was lost in PrP-null mice as suggested by Keshet et al, the predicted effect would be a reduction in superoxide production by Complex I and therefore this does not provide a possible explanation for the observed increase in superoxide formation in PrP-null mice. In addition nitric oxide promotion of superoxide formation by Complex I is dependent upon inhibition of Complex I (Riobo et al., 2001), and therefore altered nitric oxide levels would not be expected to alter Complex I superoxide production under rotenone or KCN inhibition.

Increased superoxide production by the respiratory chain is observed when increased levels of Bax in the presence of a BH3 cell death domain peptide (Starkov et al., 2002). PrP<sup>C</sup> has been suggested to inhibit Bax via Bcl-2 like activity (Bounhar et al., 2001). Therefore loss of PrP<sup>C</sup> in PrP-null mice could reasonably be expected to increase Bax levels, resulting in increased superoxide production by the respiratory chain. However the effect of Bax upon superoxide production by the respiratory chain is dependent upon the loss of cytochrome c from the mitochondria and occurs either due to a shift in the redox state of the respiratory chain, or a loss of cytochrome c scavenging of superoxide (Starkov et al., 2002). As the increase in superoxide production in PrP-null mice observed in this study occurred under inhibition of Complex I, Bax inhibition of the respiratory chain cannot explain the increased superoxide production observed in PrP-null mice, and washout of the mitochondrial matrix may remove Bax and thereby prevent it from modulating superoxide production.

Ethanol at millimolar concentrations has been determined to increase superoxide production by the respiratory chain, and a non-significant increase in superoxide
production by Complex I when mitochondria under rotenone inhibition were treated with ethanol (Bailey et al., 1999). As rotenone delivery required the use of an ethanol vehicle all samples within our study incorporated an ethanol addition to concentrations similar to those used in the study of Bailey et al, and therefore ethanol may have influenced the rates of superoxide production reported in our study. However as ethanol was added in equal concentrations to wild-type and to PrP-null samples, it would be necessary for PrP-null SMP preparations to show increased ethanol-mediated increases in superoxide radical production in order to produce the observed difference between genotypes.

Therefore whilst other groups have demonstrated a number of factors of different factors to be capable of increasing superoxide production by the respiratory chain, most operate via modulation of respiratory rates and could therefore not account for the observed differences between PrP-null and wild-type preparations.

5.27: Alteration in Complex I or Complex I subunit expression and superoxide production:

The modulators of Complex I superoxide described previously predominantly operate via inhibition of the electron transport chain, and therefore are unlikely to result in increased superoxide production when the respiratory chain is inhibited. Instead an upregulation of Complex I, or Complex I subunit(s) may be proposed to underlie the observed increase in Complex I superoxide production.

Whilst increased expression of Complex I in its entirety provides a convenient explanation for the increased superoxide production rates by PrP-null SMPs, the study of Miele et al only observed a subtle alteration in transcription of a single subunit of Complex I, NADH dehydrogenase B14.5b (Miele et al., 2002). This brings into question whether the altered superoxide production rates observed in this study were due to upregulation of the entire Complex, or due to modulation of Complex I by the altered expression of a subunit. Whilst the study of Miele et al only covered approximately two
thirds of the genome, it appears unlikely that all other genes encoding subunits of Complex I would be within the portion of the genome which was not assessed.

The simplest scenario is that all of Complex I is upregulated in PrP-null animals. This would result in greater number of the superoxide generating sites, proposed to be either an iron-sulphur or semiquinone group (Herrero & Barja, 2000), which would result in increased superoxide production upon inhibition of the respiratory chain. Such a mechanism is supported by the results of a previously published study. Hydrogen peroxide production from Complex I of isolated mitochondria from rat heart was increased relative to isolated mitochondria from pigeon heart when Complex I substrates were provided and Complex I inhibited with rotenone (St-Pierre et al., 2002). This increased superoxide production under rotenone blockade was determined to be due to increased expression of Complex I in rat heart, and following normalization of superoxide production to Complex I expression no difference between the species was detectable (St-Pierre et al., 2002). However the upregulation of Complex I in its entirety would require the DDRT-PCR reaction conducted by Miele et al to have failed to detect altered transcription of other Complex I subunits.

The upregulated subunit (NADH dehydrogenase B14.5b) does not contain any of the postulated sites of superoxide production in Complex I (Arizmendi et al., 1992). Therefore its upregulation in isolation cannot explain increased superoxide production via increased availability of superoxide production sites. An alternative mechanism is that an increase in the kinetics of superoxide production from Complex I in PrP-null SMP suspension is responsible for the observed result. Under rotenone inhibition rates of superoxide production are vastly lower than maximal electron transport rates and rates of superoxide production vary linearly with oxygen tension (Turrens et al., 1982) the rate limiting step for superoxide formation can be ascertained as the reaction between the superoxide generating charge carrier and molecular oxygen. Therefore a kinetic alteration in electron flow through Complex I to the point of inhibition is unlikely to result in an alteration in the rate of superoxide formation. An alternative
mechanism is that increased expression of NADH dehydrogenase B14.5b results in an alteration of Complex I structure resulting in an increased ability to generate superoxide radicals. This would require for either the reactivity of the responsible centre with oxygen to be increased, or for oxygen access into Complex I to be increased.

In summary our data suggests that the increased superoxide production by Complex I is due to either upregulation of Complex I in its entirety, or is due to the upregulation of the Complex I subunit NADH dehydrogenase B14.5b and possibly other subunits undetected by Miele et al which results in an alteration which increases the propensity of Complex I to generate superoxide radicals. The possibility of altered transcription of only some subunits of a respiratory Complex resulting in alteration in the activity of the entire Complex is supported by data reported in this thesis (Section 6.9) indicating that upregulation of Uqcrcl can result in increased rates of electron transport through Complex III.

5.28: Superoxide production during normal operation?
Whilst an increase in superoxide production by Complex I was observed in PrP-null SMP preparations when the respiratory chin was inhibited by either rotenone or KCN, this situation is considerably abstracted from the normal physiological state of mitochondria. In vivo mitochondria exist in a state between State 3 and State 4 (Nicholls & Ferguson, 2002). Mitochondria have been demonstrated to produce superoxide radicals under State 4 conditions (Kudin et al., 2004), but also produce superoxide when fully uncoupled with FCCP (Starkov & Fiskum, 2003), a condition similar to State 3 respiration. Therefore experimental evidence suggests that, at least in isolated mitochondria, some degree of superoxide production will occur when mitochondria respire normally.

Superoxide production by Complex I is governed by the laws of mass action – superoxide production increases with oxygen availability and with the number of reduced charge carriers acting as electron donors (Turrens et al., 1982). Assuming
similar levels of reduction of the respiratory chain, increased expression of Complex I will result in increased availability reduced charge carriers and therefore increase superoxide production. As mitochondrial membrane potential has been shown to be unchanged in PrP-null mouse brain (Lobao-Soares et al., 2005), the redox state of the electron carriers of the respiratory chain will be similar in both genotypes. Therefore it is likely that Complex I will generate more superoxide radicals in PrP-null animals when mitochondrial respiration is in a physiological state. Unfortunately however, no published study has confirmed this hypothesis by determining whether Complex I expression alters Complex I superoxide production under coupled conditions, although scope exists for manipulating Complex I subunit expression in cell lines and measuring respiration in permeabilized cells.

Another published study has found that Complex I is intrinsically susceptible to superoxide production when it becomes partially inhibited. Superoxide production from Complex I was found to occur when the Complex was inhibited 16%, whilst it was necessary to inhibit Complex III by 71% to detect superoxide production (Sipos et al., 2003). Increased electron transport activity through Complex I without upregulation of downstream components may result in Complex I effectively entering a 'more inhibited' state in PrP-null mice, and therefore will produce elevated levels of superoxide radicals. The fact that the study of Sipos et al also indicates the respiratory chain is more resistant to alterations in Complex III activity, suggests that perhaps the alteration in Complex III activity in MeCP2-null mice reported in this thesis (see Section 6.7) will be of less consequence with respect to physiological superoxide production when compared to the alteration in Complex I observed in PrP-null mice.

5.29: Superoxide production by the respiratory chain in vivo?

This thesis has reported data indicating an increased capacity for superoxide production from Complex I of the respiratory chain in PrP-null mouse brain relative to wild-type controls. However there is still uncertainty regarding the importance of superoxide production by the mitochondrial respiratory chain in vivo. The main concerns regarding
mitochondrial production of superoxide centre upon the use of isolated preparations to measure production of reactive oxygen species, which are considerably abstracted from their normal physiological environment. In particular in vitro assessment of hydrogen peroxide or superoxide production by the respiratory chain is carried out under higher oxygen concentrations than those that occur in vivo, under increased substrate concentrations and isolated mitochondria may have a greater membrane potential than in vivo. All these factors may lead to overestimation of mitochondrial superoxide production in vivo (Nohl et al., 2005). Despite this there is considerable evidence implicating mitochondrial dysfunction and oxidative stress in neurodegenerative disease.

5.30: Complex I superoxide production and mitochondrial morphology:
Data has recently been published which may implicate the observed increase in superoxide production from Complex I with the mitochondrial morphological abnormalities previous reported in this thesis. Rotenone treatment of mitochondria has been shown to promote mitochondrial outgrowth and increased branching in cultured skin fibroblasts, an effect which was controlled by Complex I superoxide production rather than inhibition of Complex I activity (Koopman et al., 2005). Despite Koppman et al detecting mitochondrial morphological abnormalities distinct from those observed in this study, their findings indicate that mitochondrial morphology can be affected by respiratory chain superoxide formation, and therefore there may be a link between the abnormal mitochondrial morphology and increased superoxide production observed in this study.

5.31: Evidence of Oxidative Stress in PrP-null mice and transmissible spongiform encephalopathies:
The finding that Complex I of the mitochondrial electron transport chain has a greater capacity to produce superoxide radicals in PrP-null mice agrees with previous studies suggesting oxidative stress in PrP-null mice. Such evidence includes that PrP-null cultures show a greater sensitivity to oxidative stress (Brown et al., 2002), increased levels of markers of oxidative damage (Wong et al., 2001b) and altered SOD activity
Previously experimental evidence suggested that a loss of antioxidant activity may underlie the state of oxidative stress in PrP-null mice. However our finding of increased superoxide production by Complex I in PrP-null mice suggests that increased superoxide production by the mitochondria may also contribute to the state of oxidative stress. Our finding also has links to evidence of oxidative stress in various TSE's, including increased levels of lipid peroxidation (Kim et al., 2000; Wong et al., 2001a) and increased protein nitration and carbonyl formation (Wong et al., 2001a) in scrapie infected mice.

5.32: Future directions for EPR investigation of respiratory chain superoxide production:

The current study has revealed a significant increase in superoxide production by Complex I in PrP-null mice. However to assess superoxide production rates it was necessary carry out the experiments using SMPs to prevent antioxidant interference. As SMPs were uncoupled superoxide production rates could only be measured following inhibition of the respiratory chain, a situation abstracted from the normal physiological scenario.

A better assessment of superoxide production by the electron transport chain would be measurement of superoxide radical production in intact mitochondria under coupled conditions. As SOD enzymes competing with tempone-H for superoxide appeared to be the problem in the current study, two ways can be envisaged by which superoxide production by intact mitochondria could be detected using EPR in the future. Currently no selective pharmacological inhibitor for Mn SOD exists. However development of a Mn SOD inhibitor which does not affect the respiratory chain may permit direct detection of superoxide production in intact mitochondria. Alternatively the development of a spin trapping compound considerably more reactive with superoxide radicals, but retaining its selectivity, may result in the spin trap effectively out-competing SOD for superoxide. A new hydroxylamine spin trap (CM-H) which has a higher rate constant with superoxide has been reported (Fink & Dikalov, 2002) and
become commercially available since this study commenced. However whilst the rate constant of CM-H with superoxide is three times that of tempone-H, it is unlikely that this increase is sufficient to significantly out-compete SOD for superoxide and therefore aid in the study of superoxide production by intact mitochondria.

Tempone-H has been reported as being able to cross biological membranes (Dikalov et al., 1997a) thereby preventing determination of the topology of superoxide production in the current study. Membrane impermeable hydroxylamine spin traps have been characterized (Dikalov et al., 1998) and have become commercially available and provide opportunities for allow determination of the topology of superoxide production by the respiratory chain. However SMP preparations have been reported to be considerably contaminated with unsealed vesicles, right side out vesicles and membranous sheets (Harmon, 1987), thereby suggesting that intact mitochondrial preparations would be more suitable for such a study although antioxidant interference could be problematical when using such a system.

5.33 Summary of results of EPR study of mitochondrial antioxidants:

Incubation of tempone-H with mitochondrial suspension was found to result in an inhibition of the background auto-oxidation signal. As this EPR signal inhibition was saturable at 50% of the auto-oxidation rate, and was not evident in Submitochondrial Particle (SMP) suspensions demonstrated to be devoid of SOD activity, it was deemed that SOD activity within the mitochondrial suspension was responsible for this inhibition of tempone-H auto-oxidation.

Upon incubation of tempone-H or CP-H with mitochondrial suspension an inhibition of the EPR signal to a level below that of the control auto-oxidation rate was observed. As the inhibition of auto-oxidation was saturable at a level approximately 50% of that of auto-oxidation, the effect was attributed to SOD activity within the mitochondrial suspension. As the majority of experiments were performed using concentrations of mitochondria that produced almost 50% inhibition of auto-oxidation, it was not possible
to compare SOD activity between PrP-null and wild-type mitochondrial suspensions using this data.

Incubation of tempone with mitochondrial suspensions produced an inhibition of the EPR signal proportional to the amount of mitochondrial protein present. As SOD is incapable of converting tempone to an EPR silent compound, this effect was attributed to low molecular weight antioxidants contained within the mitochondrial suspension reducing tempone to EPR silent tempone-H.

In an attempt to further characterize this antioxidant effect, mitochondria were treated with compounds to try and inhibit the antioxidant effect. Following treatment of mitochondrial suspensions with hydrogen peroxide the EPR signal inhibition was attenuated by approximately 80%. This indicates that the antioxidant effect of mitochondrial suspensions can be significantly, but not completely, attenuated by treatment with hydrogen peroxide. To determine if glutathione was responsible for the observed inhibition of tempone EPR signals, mitochondrial suspensions were incubated with CDNB to remove glutathione. As the mitochondrial antioxidant effect was inhibited by only 20% following this treatment, glutathione is not responsible for the majority of the observed tempone signal inhibition.

5.34: Use of EPR spectroscopy in novel assays of antioxidant activity:

The results of experiments investigating the feasibility of detecting superoxide from the respiratory chain in suspensions of intact mitochondria produced effects dominated by antioxidative, rather than pro-oxidative, activity. This indicates that the use of tempone/tempone-H and EPR may provide a number of novel approaches towards assessment of antioxidant activity of biological preparations.

The inhibition of tempone-H/CP-H auto-oxidation by SOD activity in mitochondrial suspensions indicates the potential for an EPR based assay of SOD activity which initially appears good in theory. As tempone-H/CP-H auto-oxidation is only partly
superoxide mediated and under appropriate conditions relatively low, an exogenous source of superoxide would be required to permit accurate assessment of SOD activity. The sensitivity, membrane permeability of spin traps and ease of performing this technique suggests that it may be a useful adjunct to the standard spectrophotometric assays usually employed for assay of SOD activity. However the major drawback of such a technique is the potential for considerable interference by transition metals.

The sensitivity of tempone to reduction by biological antioxidants means that assessment of tempone EPR signal loss may provide a technique to assess antioxidant capacity. This technique requires the use of tempone reduction, rather than tempone-H auto-oxidation. This is advantageous because it prevents variability due to tempone-H auto-oxidation rates and because it eliminates the influence of SOD activity. The use of EPR to detect antioxidant signals using tempone reduction appears to produce reliable data as the tempone reducing effect is linear with quantity of tissue added. However the failure to determine the specific antioxidants implicated in tempone reduction, in conjunction with the variable stoichiometry of antioxidants reacting with tempone (~1.6 for ascorbate, ~1 for GSH) mean that caution should be employed if antioxidant capacity is studied using such a technique.
Chapter 6:

Mitochondrial morphology and respiration rates in MeCP2-null mice

—a model of Rett Syndrome
Background:

6.1: Mitochondrial abnormalities in Mecp2-null mice:
Mitochondrial abnormalities were first identified in Rett Syndrome patients a considerable time ago (Ruch et al., 1989; Eeg-Olofsson et al., 1990; Dotti et al., 1993), and more recently abnormalities in the expression of genes encoding mitochondrial components have been identified (Kriaucionis et al., 2006). This Chapter will employ the techniques previously used to study PrP-null mice in Chapter 4 to investigate whether mitochondria isolated from Mecp2-null mouse brain (a model of Rett Syndrome) display abnormalities in terms of their morphology or respiratory activity. The aims of this Chapter are therefore two-fold. Firstly this study will investigate the hypothesis that mitochondrial structure and function is altered in Mecp2-null mice brain tissue, thereby giving insight into the cellular defects underlying Rett Syndrome. And secondly the results may provide an insight as to whether the alterations in mitochondrial structure and function presented in Chapter 4 of this thesis are a specific feature of PrP-null mice, or rather are a general indicator of mitochondrial dysfunction.

Analysis of gene expression by other groups, in conjunction with the analysis of mitochondrial respiration presented in this thesis, has suggested that upregulation of a single respiratory complex subunit may result in altered mitochondrial respiration rates through the entire respiratory chain. Such observations have been made in both PrP-null mice (Miele et al., 2002) and Chapter 4) and in Mecp2-null mice ((Kriaucionis et al., 2006) and results to be presented in this Chapter). Therefore the respiration rates of an N2A cell line overexpressing Uqcrcl (Kriaucionis et al., 2006) will be assessed in order to test this hypothesis.
Methods:

6.2: Experimental animals and preparation of mitochondrial suspensions:

*Mecp2*-null mice were generated by crossing heterozygous female mice with wild-type male mice (Guy et al., 2001). The resulting male offspring were genotyped and *Mecp2*-null mice were compared to wild-type littermate controls, heterozygous females were used to maintain the colony. This technique of maintaining the colony is superior that used for the study of *PrP*-null mice, where separate homozygous lines were maintained.

Mitochondria were isolated from the brain tissue as previously described in Section 4.1, employing the digitonin purification technique. As the time of symptom onset is variable between *Mecp2*-null animals, animals were selected for the symptomatic arm of the study upon their presentation of symptoms and were compared to wild-type control littermates. Symptomatic animals displayed obvious symptoms of tremor and inertia and, in many cases, limb clasping. These symptoms tended to manifest when the animals reached approximately 2 months of age. For the presymptomatic study *Mecp2*-null animals and wild-type control littermates were utilized prior to the acquisition of symptoms, at around 1 month of age.

6.3: Electron microscopy:

Mitochondrial suspensions from brain tissue of fully symptomatic *Mecp2*-null and age-matched littermate control animals were prepared for electron microscopy according to the protocols outlined in Section 4.3. Sections were examined using a Philips CM120 Biotwin transmission electron microscope by Dr Skirmantas Kriaucionis. Analysis of mitochondrial diameter, Section area, perimeter and cristae density was carried out as described in Section 4.4.

6.4: Measurement of mitochondrial respiration rates:

The oxygen consumption rates of isolated mitochondria from *Mecp2*-null and wild-type control mice were measured using a Clark Oxygen Electrode using the protocols described in Section 4.10.
6.5: Oxygen consumption rates of a Uqcrcl over-expressing cell line:

A Uqcrcl-overexpressing N2A cell line was created by Dr Skirmantas Kriaucionis (Kriaucionis et al., 2006) using the following technique. Mouse Uqcrcl CDS was PCR amplified from cDNA (Clontech, MARATHON ready mouse 11d embryo cDNA) and cloned into a pBABE Puro (EcoRI, Sall) plasmid. The resulting clone was fully sequenced and transfected (Ca phosphate) into Phoenix Eco retrovirus packaging cell line. The viral supernatant was collected two days after transfection and applied onto N2A cells together with final concentration of 4 µg/ml of polybrene. Puromycin to a concentration of 3µg/ml was applied 48h after infection and maintained during culturing of cells. A control cell line was prepared simultaneously by infecting N2A cells with the vector alone. N2A cells were cultured in DMEM + 10% FCS + Non essential amino acids (Invitrogen) and kept at 37°C. Medium was replaced with puromycin free medium 24h before respiration measurements.

Mitochondrial oxygen consumption was measured in permeabilised neuroblastoma (N2A) cells essentially by the method of Hofhaus et al (Hofhaus et al., 1996). Cells were harvested by trypsinisation, diluted 1 to 5 in medium A (250 mM sucrose, 10 mM MgCl₂, 20 mM HEPES, pH7.1) and spun down at 250 g for 2 minutes. Cells were resuspended in 1ml medium A to which 1 ml of 200 µg/ml digitonin was added. Following incubation at 37°C for 1 minute, the cells were diluted 1 in 10 dilution in medium A and spun down at 250 g for 3 minutes. The final pellet was resuspended in 1 ml medium A and triturated using 5 passes through a fire-polished glass pipette to separate the cells. Cell counts were performed in triplicate for each sample using a haemocytometer. Cell viability was tested using Trypan Blue. Typically >95% of cells stained with Trypan Blue following digitonin permeabilisation. 4x10⁶ permeabilised cells were introduced to the electrode chamber in a final volume of 3 ml in N2A respiration buffer (medium A supplemented with 1 mM ADP and 2 mM KH₂PO₄). Following acquisition of a stable baseline, Complex I substrates pyruvate and malate were added (to a concentration of 5 mM and 2.5 mM respectively), before Complex I was inhibited by addition of rotenone (2 µM). The Complex II substrate succinate was
then added (15 mM) and respiration measured before maximal inhibition of the chain at Complex III using myxothiazol (50 nM). Finally the Complex IV substrate TMPD (80 μM) was added together with ascorbate (10 mM) and a rate determined prior to complete inhibition of the chain at Complex IV by addition of KCN (1 mM).
Results:

6.6: Analysis of mitochondrial size by electron microscopy in Mecp2-null mice:
Mitochondrial morphological abnormalities have long been observed in Rett syndrome (Ruch et al., 1989; Eeg-Olofsson et al., 1990), although contradictory reports exist (Nielsen et al., 1993). Mitochondria from Mecp2 null mice, which have been proposed as a model of Rett syndrome (Guy et al., 2001; Chen et al., 2001), were examined using electron microscopy to determine if they were morphologically abnormal.

As Mecp2-null mice are asymptomatic for up to 8 weeks (Guy et al., 2001), to determine if mitochondrial morphological abnormalities played a role in symptom acquisition the mitochondria analysed were from fully symptomatic Mecp2-null animals and were compared to age-matched wild-type littermate controls.

Figure 6.6.1: Electron micrographs of mitochondria from a Mecp2-null preparation (KO) and a littermate wild-type control (WT). Structures within both preparations appear to be predominantly mitochondrial. Scale bar equals 500nm.

Structures were predominantly identified as mitochondria and mitochondrial structure was clearly visible (Figure 6.6.1). Structures were identified as mitochondria due to the presence of an outer membrane and cristae. Using ImageJ software, mitochondria were
highlighted following their outer membrane and their area, perimeter and Feret’s Diameter were measured.

Figure 6.6.2: Box plots comparing mitochondrial parameters of Mecp2-null preparations (light grey) against wild-type controls (dark grey). Panel A shows data for mitochondrial diameter, panel B shows data for mitochondrial perimeter and panel C shows data for mitochondrial area. Data consists of analysis of 201 wild-type mitochondria and 247 Mecp2-null mitochondria each approximately evenly spread across mitochondrial preparations from 2 wild-type and 3 Mecp2-null animals. Data was log_{10} transformed to conform to normality and equal variance, and statistics were performed as 1-way ANOVAs – no statistically significant differences (P>0.05) were observed for any of the parameters analysed. Box plots demonstrate the median (line), 25th to 75th percentile (box), 10th to 90th percentile (bars) and 5th to 95th percentile (dots).
Figure 6.6.2 displays a summary of the analysis of mitochondrial size parameters for MeCP2-null mice and their wild-type controls. Panel A demonstrates that no significant difference was detected in mitochondrial diameters between the genotypes (Wild-type mean 0.295μm ± 0.006 SEM n = 201, MeCP2-null mean 0.309μm ± 0.006 SEM n = 247, \( P > 0.05 \)). Panel B demonstrates that no significant difference was detected between mitochondrial perimeters between genotypes (Wild-type mean 0.794μm ± 0.017 SEM n = 201, MeCP2-null mean 0.827μm ± 0.016 SEM n = 247, \( P > 0.05 \)). Panel C demonstrates that no significant difference was detected between mitochondrial Section areas between genotypes (Wild-type mean 0.0653 ± 0.0030 SEM n = 201, MeCP2-null mean 0.0702μm² ± 0.0025 SEM n = 247, \( P > 0.05 \)). Taken together this data suggests that mitochondrial size is not significantly altered in the MeCP2-null preparations when compared to wild-type controls.

A considerably lower number (approximately 50%) of mitochondria were analysed for the MeCP2-null study than were assessed in the PrP-null study. However it must be noted that when similar numbers of mitochondria were assessed in the PrP-null study a highly significant difference was observed between the genotypes. As the degree of scatter of the data in the MeCP2-null data is similar to that in the PrP-null study, and because the mean mitochondrial size parameters between genotypes are within 5% of one another for perimeter and diameter, and 10% of each other for area, it is unlikely that a large difference in mitochondrial size exists between the genotypes, and certainly any difference would be considerably smaller than was seen in the PrP-null study. It was not possible to thoroughly analyse mitochondrial parameter data in the MeCP2-null study for multimodal distribution due to this lower \( n \) number.
6.7: Analysis of mitochondrial cristae density using electron microscopy in Mecp2-null mice:

![Box plots showing cristae measurements for wild-type (dark-grey) and Mecp2-null (light-grey) mitochondrial preparations.](image)

Figure 6.7.1: Box plots showing cristae measurements for wild-type (dark-grey) and Mecp2-null (light-grey) mitochondrial preparations. Panel A compares cristae density expressed as a percentage of the mitochondrial interior between Mecp2-null animals and wild-type controls. Panel B compares cristae area within mitochondrial Sections between Mecp2-null animals and wild-type controls. Data consists of analysis of 169 wild-type and 193 Mecp2-null mitochondria, spread approximately evenly across 2 and 3 preparations for wild-type and Mecp2-null respectively. Statistics for Panel A were performed as 1-way ANOVA on raw data, for Panel B data was log_{10} transformed to conform to normality and equal variance prior to statistical testing using 1-way ANOVA. No statistically significant difference between genotypes was detected for either parameter assessed. Box plots demonstrate the median (line), 25th to 75th percentile (box), 10th to 90th percentile (bars) and 5th to 95th percentile (dots).

The data in Figure 6.7.1 panel A demonstrates that no significant difference was detected between the percentage cristae density between Mecp2-null mitochondria and wild-type controls (wild-type mean 58.4% ± 1.3 SEM n = 169, Mecp2-null mean 58.8% ± 1.3 SEM n = 193, P > 0.05). Despite the lack of significant differences between mitochondrial section area or percentage cristae density, mitochondrial cristae area was
assessed as non-significant changes in section area and cristae density may summate to produce a significant difference in total cristae area. However the data in Figure 6.7.1 panel B demonstrates that no significant difference in mitochondrial section cristae area was observed between genotypes (wild-type mean $0.0280 \mu m^2 \pm 0.0013$ SEM $n = 169$, Mecp2-null mean $0.0295 \mu m^2 \pm 0.0011$ SEM $n = 193$, $P > 0.05$).

Therefore analysis electron microscopy of mitochondria from symptomatic Mecp2-null mice has failed to detect significant difference from control animals. As an identical protocol was employed to analyse PrP-null mitochondria and successfully identified a clear mitochondrial morphological difference, it is unlikely that the failure to detect a difference in Mecp2-null animals is due to abnormal mitochondria being lost at some stage of sample preparation.

6.8: Analysis of oxygen consumption rates by mitochondrial suspensions prepared from symptomatic Mecp2-null mice:

A mitochondrial abnormality(s) has long been postulated as a causative or contributing factor towards Rett Syndrome (Eeg-Olofsson et al., 1990). Although no morphological abnormalities were detected in the brain of Mecp2-null mice (a model of Rett Syndrome) using electron microscopy, recent experiments on this mouse model have uncovered alterations in gene transcription of mitochondrial components (Kriaucionis et al., 2006). Therefore a thorough investigation of the respiratory chain of these mice therefore was carried out using an oxygen electrode. Oxygen consumption rates of isolated mitochondria from Mecp2-null mice exhibiting symptoms were compared to wild-type age-matched litter-mate controls.
Table 6.8.1: Oxygen Consumption Rates of Mitochondrial Suspensions from symptomatic MeCP2-null mice and age-matched wild-type control mice. Rates are expressed as nmoles oxygen per minute per milligram protein. A natural log transform was applied to all the data to make it conform to normality and equal variance.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type (Mean ± SEM)</th>
<th>MeCP2-null (Mean ± SEM)</th>
<th>P-value 2-Way ANOVA Tukey Test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>State 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>15.4 ± 0.7</td>
<td>20.4 ± 0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Succinate</td>
<td>41.7 ± 1.8</td>
<td>49.5 ± 1.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TMPD/Ascorbate</td>
<td>76.1 ± 4.2</td>
<td>75.2 ± 5.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>State 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>77.6 ± 1.9</td>
<td>88.8 ± 2.0</td>
<td>&gt;0.05*</td>
</tr>
<tr>
<td>Succinate</td>
<td>97.5 ± 2.9</td>
<td>107.4 ± 2.9</td>
<td>&gt;0.05*</td>
</tr>
<tr>
<td>TMPD/Ascorbate</td>
<td>97.8 ± 6.6</td>
<td>97.1 ± 7.1</td>
<td>&gt;0.05*</td>
</tr>
<tr>
<td><strong>State 4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>18.3 ± 0.7</td>
<td>22.5 ± 0.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Succinate</td>
<td>45.8 ± 1.6</td>
<td>56.3 ± 1.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TMPD/Ascorbate</td>
<td>73.0 ± 4.0</td>
<td>78.6 ± 6.1</td>
<td>&gt;0.05</td>
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<tr>
<td><strong>FCCP Uncoupled</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>77.7 ± 2.9</td>
<td>86.8 ± 2.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Succinate</td>
<td>80.6 ± 3.4</td>
<td>93.0 ± 3.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TMPD/Ascorbate</td>
<td>80.9 ± 6.0</td>
<td>84.5 ± 5.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>RCR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>4.3 ± 0.1</td>
<td>4.0 ± 0.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.1 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>ATP:O</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

* Data could not be transformed to conform to normality and equal variance – a non-parametric Friedman Test with Conover Multiple Comparison test was employed.
The data in Table 6.8.1 shows that the maximal uncoupled rates of respiration observed following FCCP addition were significantly increased in the Meep2-null mice when mitochondria were provided with pyruvate/malate or succinate. However when the substrate TMPD/ascorbate was used to energize mitochondria, no significant difference between the genotypes was observed. As pyruvate/malate metabolism results in electron flow through Complexes I, III and IV, whilst succinate metabolism results in electron flow through Complexes II, III and IV, the alteration between the genotypes is localized to somewhere within Complexes III and IV. TMPD/ascorbate produces electron flow through only Complex IV, hence isolating the change to Complex III. This finding is supported by the data for State 3 respiration – although the difference is non-significant due to the necessity to use a less powerful non-parametric statistical test for two-way analysis, a strong trend towards an increase in respiration rates, of similar magnitude to the FCCP difference, was observed with Complex I and II substrates. Indeed these increases in State 3 respiration rates were significant when parametric 1-way analysis of the data was performed. This would suggest that the change is somewhere within the respiratory chain rather than an alteration in the activity of ATP synthase.

The data for State 3 oxygen consumption rates, like the State 3 data for the aged PrP-null animals, was unable to be transformed to conform to normality and equal variance. Although State 3 respiration approximately mirrors the FCCP uncoupled data in these experiments, the inability to transform the data for these states alone perhaps indicates a subtle modulation of respiratory rates by ATP synthase. The lack of difference in the ATP:O ratio between genotypes suggests that there is no change in the stoichiometries of protons transported per electron flowing down the respiratory chain, or the efficiency of ATP synthase.

The rates of coupled respiration (States 2 and 4), when the proton translocation associated with Complexes I, III and IV works against an electrochemical proton gradient, showed highly significant increases in the symptomatic Meep2-null mitochondria when compared to wild-type controls when substrates were fed in at
Complex I or II. No difference in coupled respiration was observed when mitochondria were provided with Complex IV substrate. Coupled respiration is dependent upon the degree of non-Ohmic proton conductance across the inner mitochondrial membrane, and normally is not associated with changes in activity of respiratory Complexes. This may make the lack of difference in coupled respiration when using Complex IV substrates initially surprising, but the fact that the mitochondria are almost completely uncoupled (i.e. the chain is operating at almost maximal capacity to maintain a proton gradient) when using these substrates could explain the lack of an increase in coupled respiration in the MeCP2-null animals. These increases in coupled respiration describe a scenario whereby the inner mitochondrial membrane has an increased background ‘leak’, resulting in the respiratory chain having to operate faster to maintain a proton gradient, and hence consuming more oxygen. The result of this is that the mitochondria work less efficiently as more substrate is oxidized in order to maintain a proton gradient resulting in a lesser fraction being used to phosphorylate ADP.

As the increases in State 3 and State 4 respiration are antagonistic when determining the RCR, the direction of change of RCR cannot be predicted simply by the direction of the changes in State 3 and 4. When the RCR’s were calculated a significant decrease in the RCR was observed when mitochondria were provided with either pyruvate/malate or succinate, indicating that the increase in State 4 respiration dominates over the increase in State 3. Overall the mitochondria from symptomatic mutants have an overall greater respiratory capacity (State 3 and uncoupled rates) but also have a reduction in respiratory efficiency (State 2 and 4).

6.9: Analysis of oxygen consumption rates by mitochondrial suspensions prepared from presymptomatic MeCP2-null mice:
As a clear mitochondrial abnormality was detectable in MeCP2-null animals following the onset of symptoms, further experiments was carried out to determine if the onset of the mitochondrial abnormality correlated with the onset of symptoms.
Table 6.9.1: Oxygen Consumption Rates of Mitochondrial Suspensions from presymptomatic MeCP2-null mice and age-matched wild-type control mice. Rates are expressed as nmoles oxygen per minute per milligram protein. A natural log transform was applied to all the data to make it conform to normality and equal variance.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type (Mean ± SEM)</th>
<th>n</th>
<th>MeCP2-null (Mean ± SEM)</th>
<th>n</th>
<th>P-value 2-Way ANOVA</th>
<th>Tukey Test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>State 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>22.3 ± 0.8</td>
<td>8</td>
<td>21.6 ± 1.5</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>50.0 ± 2.3</td>
<td>8</td>
<td>50.0 ± 2.0</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>TMPD/Ascorbate</td>
<td>88.4 ± 3.5</td>
<td>8</td>
<td>80.9 ± 2.9</td>
<td>7</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td><strong>State 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>83.3 ± 4.6</td>
<td>8</td>
<td>84.0 ± 4.3</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>110.3 ± 4.7</td>
<td>8</td>
<td>109.7 ± 4.2</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>TMPD/Ascorbate</td>
<td>105.7 ± 3.9</td>
<td>8</td>
<td>97.0 ± 5.5</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td><strong>State 4</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>25.8 ± 1.3</td>
<td>8</td>
<td>25.7 ± 1.5</td>
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<td>&gt;0.05</td>
<td></td>
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<tr>
<td>Succinate</td>
<td>61.3 ± 2.2</td>
<td>8</td>
<td>60.8 ± 1.5</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>TMPD/Ascorbate</td>
<td>85.4 ± 2.9</td>
<td>8</td>
<td>77.9 ± 4.0</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td><strong>FCCP Uncoupled</strong></td>
<td></td>
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</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>82.3 ± 4.5</td>
<td>8</td>
<td>85.5 ± 2.8</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>104.7 ± 3.9</td>
<td>8</td>
<td>102.7 ± 3.2</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>TMPD/Ascorbate</td>
<td>82.1 ± 3.5</td>
<td>8</td>
<td>73.3 ± 4.1</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td><strong>RCR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>3.2 ± 0.1</td>
<td>8</td>
<td>3.3 ± 0.1</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>1.8 ± 0.1</td>
<td>8</td>
<td>1.8 ± 0.1</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td><strong>ATP:O</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>2.5 ± 0.1</td>
<td>8</td>
<td>2.6 ± 0.1</td>
<td>8</td>
<td>&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

The data presented in Table 6.9.1 show that the differences detected in the respiration rates of symptomatic animals compared to wild-type controls were not evident in those
animals prior to the acquisition of symptoms. These animals failed to show a Complex III specific increase in uncoupled respiration, or any difference in rates of coupled respiration. This would imply that the mitochondrial respiration differences reported appear at around the time of onset of symptoms, although whether these differences are cause or a consequence of the symptoms remains enigmatic.

The data also show that respiration rates were universally higher in the presymptomatic group than in the symptomatic group. Obviously the experiments examining the presymptomatic animals had to be performed upon animals of a considerably younger age than in the symptomatic experiments (presymptomatic wild-type 34.5 days ± 3.2 SEM Mecp2-null 33 days ± 3 SEM n = 4, symptomatic wild-type 59.1 days ± 3.4 SEM Mecp2-null 61.8 days ± 2.2 SEM n = 8) and, as an identical isolation procedure was employed for both parts of the study, it is possible that this introduced an artefact into the study due to altered mitochondrial purity or uncoupling of the mitochondria during isolation. It is, however, unlikely that a preparation artefact resulting decreased coupling of the mitochondria would hide any increase in inner mitochondrial membrane proton leak, as the mitochondria used within the presymptomatic part of the experiment showed consistently acceptable RCR’s of >3.

6.10: Oxygen consumption rates of a Uqcrc1-overexpressing cell-line:

The mitochondrial uncoupled respiration abnormalities in Mecp2-null mouse brain could arise as a result of any one of a multitude of events. One potential mechanism is that the observed over-expression of a single subunit of Complex III (Uqcrc1) alone could be responsible for the measured increase in oxygen consumption on Complex I and II substrates. To test this hypothesis a Uqcrc1-overexpressing N2A cell line was created. A single copy of the Uqcrc1 gene was delivered into mouse N2A cells using retroviral infection, which resulted in a mild overexpression of the gene. Infected cells were maintained and expanded by selecting for the puromycin resistance gene, which was present in the delivery vector. Stable Uqcrc1 over-expressing cell line produced 2.8
times more RNA and 1.6 times more Uqcrcl protein than the control cells infected with vector only (Kriaucionis et al., 2006).

To assess the impact of Uqcrcl overexpression upon mitochondrial respiration, the rates of oxygen consumption of permeabilised cell suspensions of Uqcrcl overexpressing and sham infected control N2A cells were assessed using a Clark oxygen electrode.

Mitochondria within the permeabilised N2A cells appeared to be uncoupled as their oxygen consumption rate was increased only slightly by the addition of ADP or FCCP. A likely explanation for this uncoupling is that, following permeabilisation with digitonin, ionic gradients across the cell plasma membrane collapse resulting in continuous activity of active transporters within the plasma membrane, thus consuming ATP. A high ADP:ATP ratio is therefore maintained allowing the proton gradient across the inner mitochondrial membrane to be dissipated through ATP synthase, thus disinhibiting the ETC resulting in high levels of oxygen consumption. In order to prevent variation in the degree of mitochondrial coupling from affecting the oxygen consumption rates for permeabilised cells, the ADP:ATP ratio was maintained at a high level by the inclusion of ADP to a concentration of 1mM within the buffer.

Table 6.10.1: Oxygen consumption rates of permeabilised Uqcrcl overexpressing and control N2A cells. Rates expressed as fmoles/min/cell

<table>
<thead>
<tr>
<th></th>
<th>Sham Infected N2A cells</th>
<th></th>
<th>Uqcrcl-Overexpressing N2A cells</th>
<th></th>
<th>P-value 2-Way ANOVA Tukey Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± SEM)</td>
<td>n</td>
<td>(Mean ± SEM)</td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>Pyruvate/Malate</td>
<td>0.919 ± 0.035</td>
<td>19</td>
<td>1.111 ± 0.027</td>
<td>19</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Succinate</td>
<td>1.460 ± 0.087</td>
<td>19</td>
<td>1.723 ± 0.086</td>
<td>19</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TMPD/Ascorbate</td>
<td>2.238 ± 0.105</td>
<td>19</td>
<td>2.301 ± 0.076</td>
<td>19</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>
The data in Table 6.10.1 demonstrates that Uqcrcl-overexpressing N2A cells show a statistically significant increase in uncoupled oxygen consumption rate relative to control when substrates that enter the respiratory chain upstream of Complex III were used (pyruvate/malate, succinate) but not when substrates enter at Complex IV (TMPD/Ascorbate). This result replicates the finding that uncoupled respiration rates were increased in Mecp2-null mouse brain, and thereby suggests that Uqcrcl overexpression is causative in the production of the mitochondrial respiration abnormality. The lack of coupling of mitochondria within permeabilised N2A cells precludes the investigation of coupled respiration using this system, however it is unlikely that an increase in Uqcrcl expression would also result in permeabilisation of the inner mitochondrial membrane and hence cause the increases in States 2 and 4 respiration observed.
Discussion:

6.11: Summary of findings from the study of mitochondrial morphology in MeCP2-null mouse brain using electron microscopy:

As previous studies have suggested mitochondrial abnormalities in MeCP2-null mice and Rett Syndrome, morphological analysis of mitochondria isolated from MeCP2-null mice was performed. Analysis of mitochondrial morphology of MeCP2-null mice and wild-type littermate controls revealed no statistically significant difference in mitochondrial size or cristae density. Reassuringly mitochondrial size parameters in MeCP2-null mice and littermate controls were similar to those detected from the wild-type animals in the PrP-null study. The lack of mitochondrial structural differences in a mouse model of a disease condition considered to involve mitochondrial dysfunction indicates that the observed mitochondrial abnormalities in PrP-null mice are not a ubiquitous marker for mitochondrial dysfunction.

6.12: Other studies of mitochondrial morphology in Rett Syndrome:

Whilst no other studies of mitochondrial morphology in MeCP2-null mice have been published, numerous accounts of mitochondrial morphological abnormalities in the skeletal muscle tissue of Rett syndrome sufferers exist. Analysis of mitochondria from muscle biopsies of Rett sufferers have revealed vacuolated mitochondria (Ruch et al., 1989; Eeg-Olofsson et al., 1990) and swollen mitochondria (Dotti et al., 1993). However other groups have refuted these findings and suggest that mitochondria are morphologically normal in Rett syndrome (Coker & Melnyk, 1991; Nielsen et al., 1993), although the former of these studies did detect abnormalities in respiratory chain activity. Whilst a consensus may have been reached that mitochondrial abnormality is a feature of Rett Syndrome, the issue of whether mitochondria become structurally abnormal, and in which tissues, remains unanswered.

It is difficult to relate these studies to the observation of no mitochondrial structural abnormality in MeCP2-null mouse brain, due to the different tissues and the different species investigated. However as the mouse model does develop some of the
neurological symptoms of Rett Syndrome (Guy et al., 2001) whilst not exhibiting morphologically abnormal mitochondria in brain tissue, it can be hypothesized that gross mitochondrial structural abnormality is not required for the acquisition of the neurological symptoms of Rett syndrome.

6.13: Summary of results from the study of mitochondrial respiration of Mecp2-null mouse brain:

Whilst no difference in mitochondrial morphology was detected in Mecp2-null mouse brain, this did not preclude the possibility that mitochondrial function was altered, and so a thorough investigation of the activity of the respiratory chain in Mecp2-null mice was performed. The results presented demonstrate clear abnormalities in the respiration rates of mitochondria from brain tissue of symptomatic Mecp2-null mice relative to age and sex-matched littermate controls. Two discrete mitochondrial abnormalities were observed in Mecp2-null mice that had acquired symptoms of Rett syndrome.

Coupled respiration rates were significantly elevated by around 25% when mitochondria were provided with pyruvate/malate or succinate, but not TMPD/ascorbate, as respiratory substrates indicating an increased permeability of the inner mitochondrial membrane to protons. Whilst the lack of difference in the rates of coupled respiration when TMPD/ascorbate are provided as respiratory substrates may initially seem surprising, it should be noted that mitochondrial suspensions exhibited respiratory control ratios of approximately 1 when provided with these substrates. When the RCR approaches 1 the respiration rate is determined by maximal capacity of the respiratory Complex(es) involved and not by the permeability of the inner mitochondrial membrane to protons. Therefore whilst the permeability of inner mitochondrial membrane to protons may have increased in Mecp2-null mice, this will not equate to an increase in coupled respiration rates when provided when TMPD/ascorbate as respiratory substrates.
In addition an increase in uncoupled respiration rates was observed when mitochondria were provided with either the Complex I substrates pyruvate/malate or the Complex II substrate succinate, but not when provided with the Complex IV substrate TMPD/Ascorbate. As the electron transport pathways are discrete through Complexes I and II and converge at the level of the ubiquinone pool from where they are passed to Complex III and to Complex IV via cytochrome c (Nicholls & Ferguson, 2002), the simplest alteration in the respiratory chain capable of producing the observed effect would be an increase in respiratory capacity through Complex III. However the data does not preclude the possibility that the difference between genotypes is caused by increased respiratory capacity through both Complexes I and II.

Study of mitochondrial suspensions isolated from brain tissue of MeCP2-null mice prior to the acquisition of overt clinical symptoms revealed no significant differences in respiration rates. This suggests that the abnormality in mitochondrial respiration and clinical symptom acquisition are related in MeCP2-null mice. However the data does not indicate whether the loss of mitochondrial functionality is causative towards, or merely a consequence of, clinical symptom acquisition. In addition recent reports have suggested that MeCP2-null mice may begin to show some subtle postural and developmental abnormalities prior to weaning (Santos et al., 2006; Picker et al., 2006). The manifestation of these abnormalities prior to the age at which mice were classified as presymptomatic in the current study, and the lack of difference in mitochondrial respiration rates in these animals classified as presymptomatic, may suggest that the detected mitochondrial abnormalities are not the primary cause of the acquisition of clinical symptoms of in the MeCP2-null mouse model.

6.14: Gene Expression Analysis of MeCP2-null mice and Rett Syndrome:
In a collaborative study, Kriaucionis et al compared gene expression in MeCP2-null and wild-type mouse brain using DDRT-PCR (Kriaucionis et al., 2006). They observed an upregulation of Uqcrcl transcription, a gene encoding one of the core subunits of Complex III, by approximately 22% in early symptomatic animals which increases to
73% by the late symptomatic stage. This alteration in gene transcription was not evident in animals prior to the acquisition of symptoms. These results are in good agreement with our findings indicating an increase of respiration rates through Complex III in symptomatic, but not presymptomatic, Mecp2-null mice.

A correlation between the increased transcription of a respiratory Complex subunit and mitochondrial respiration through that Complex has been identified in Mecp2-null mice, an observation similar to that made in the study of PrP-null mouse brain mitochondria. To determine the likelihood of whether an upregulation of Complex III alone would be capable of producing an increase in the rate of respiration through the respiratory chain, it is necessary to consider the flux control coefficient of Complex III. Whilst Complex III has been reported to possess a significant flux control coefficient (approximately 0.2) in tissues such as skeletal and cardiac muscle (Rossignol et al., 2000), studies of Complex III flux control in brain tissue have produced variable results. Rossignol et al have reported a flux control coefficient of 0.02 for Complex III in isolated brain mitochondria provided with pyruvate and malate (Rossignol et al., 2000), whereas higher flux control coefficients of 0.15 for non-synaptic (Davey & Clark, 1996) and 0.20 for synaptic mitochondria (Davey et al., 1998) provided with glutamate and malate have been reported. The discrepancy between these values has been suggested to be due to differences between the substrates provided and the composition of the respiratory buffer (Rossignol et al., 2000). The isolation procedure used in this study was very similar to that used by Davey and Clark for non-synaptic mitochondria, however the use of pyruvate/malate as respiratory substrate mirrors the experiments of Rossignol et al. It is therefore difficult to predict the flux control coefficient of Complex III in the current study. However in order for Complex III upregulation to produce the observed difference in mitochondrial respiration rates, assuming the percentage increase in gene transcription detected by Kriaucionis et al (Kriaucionis et al., 2006) equates to a similar increase protein expression, a flux control coefficient approaching 0.2 would be required. However upregulation of only a single subunit of Complex III was detected, rather than upregulation Complex III in its entirety (Kriaucionis et al., 2006). To
address whether upregulation of a single subunit of Complex III could result in an alteration in respiration rates, a cell line overexpressing Uqcrcl was created and studied (see Section 6.9).

In addition to an increase in the transcription of Uqcrcl, a decreased transcription of NADH dehydrogenase subunit 2 (a subunit of Complex I) by up to 40% in late symptomatic Mecp2-null mice has also been identified (Kriaucionis et al., 2006). However the mitochondrial respiration rates from animals at this stage of disease progression were not assessed in the current study both for humane reasons and because an alteration in mitochondrial functionality at this stage of disease progression could not be considered as causative towards symptom acquisition.

The 16S rRNA subunit has been identified as mutated in about 50% of Rett syndrome sufferers and their mothers, a phenomenon which was not evident in control individuals (Tang et al., 1997). This finding suggests that abnormalities in the 16S subunit of rRNA may be a contributing factor towards Rett Syndrome. Apart from encoding a ribosomal subunit, 16S rRNA has been proposed to encode the peptide humanin (Maximov et al., 2002) which has been suggested to be protective against Alzheimer's Disease (Hashimoto et al., 2001). The findings of mutated 16S rRNA in Rett syndrome, altered 16S rRNA expression in PrP-null mice (Miele et al., 2002) and the humanin peptide protecting against Alzheimer’s Disease suggest a commonality between these disparate neurological conditions. However further study of Rett Syndrome sufferers discovered that 16S rRNA showed no mutations in a large number of Spanish Rett sufferers (Armstrong et al., 2000). This indicates that mutation of 16S rRNA is not essential for the development of Rett Syndrome.

6.15: Mitochondrial respiration in Uqcrcl overexpressing N2A cells:
To address whether the increased mitochondrial respiration rates observed in Mecp2-null mice could be directly caused by overexpression of Uqcrcl, a line of Uqcrcl overexpressing N2A cells was created and mitochondrial respiration measurements were
made. To eliminate the possibility of the viral vector altering mitochondrial respiration rates, controls cells were infected with the viral vector containing a puromycin resistance gene to permit selection for infected cells. Mitochondrial respiration rates in permeabilized cells were measured in the presence of excess ADP to ensure that mitochondria were fully uncoupled (Hofhaus et al., 1996). A statistically significant increase in respiration rate was observed in Uqcrcl overexpressing cells when provided with pyruvate/malate or succinate, but not on TMPD/ascorbate. Whilst it is not possible to directly compare respiration rates measured from permeabilised cells and isolated mitochondria due to normalization of the data to cell number and protein content respectively, the percentage increases in pyruvate/malate and succinate uncoupled respiration rates were similar between the two studies. The overall finding of the N2A study is an increase in respiratory capacity through Complex III, which suggests that upregulation of the Uqcrcl subunit of Complex III is capable of producing an increase in respiratory capacity through Complex III. The finding of this study also indicates that it is at least possible for the upregulation of a single subunit of a respiratory Complex to produce an overall increase in the activity of that Complex. This of course has particular resonance with the study of PrP-null mice reported here, where an increase in respiratory capacity through Complex I was observed in an animal model that has been shown to possess increased transcription of the Complex I subunit NADH B14.5b (Miele et al., 2002).

The finding that mitochondrial respiration rates were increased in Uqcrcl overexpressing cells was surprising as it demonstrated that upregulation of this single core subunit of Complex III could result in an overall increase in Complex III activity. Uqcrcl is a nuclear encoded protein of approximately 480 amino acids in length (Hoffman et al., 1993), and study of bovine Complex III revealed that Uqcrcl is one of the 11 polypeptides that comprise the Complex although it does not contain one of the 3 prosthetic groups within the Complex (Iwata et al., 1998). As the function of Uqcrcl within Complex III remains unresolved, it is only possible to speculate as to the mechanism whereby increases Uqcrcl expression could lead to increased Complex III
activity. The increase in Uqcrcl could induce an increase in transcription of other components of Complex III thereby increasing respiration rates through Complex III, Uqcrcl could be a rate limiting factor for Complex III activity, or Uqcrcl could bind and inactivate an inhibitor of Complex III activity. However perhaps the most compelling hypothesis as to how increases in Uqcrcl expression could lead to a increase in Complex III activity is by promoting the assembly of Complex III from its constituent polypeptides as an essential role for Uqcrcl within the assembly of Complex III has been demonstrated in yeast (Tzagoloff et al., 1986).

6.16: Mitochondrial Dysfunction and clinical symptoms of Rett Syndrome:
A number of the clinical symptoms of Rett Syndrome suggest that mitochondrial dysfunction may play a role within the disease. Clinical studies have uncovered an increase in blood and CSF levels of pyruvate and lactate in some sufferers of Rett Syndrome, although variability within each patient over time resulted in statistical significance not being reached (Haas et al., 1995). In the Mecp2-null mouse model of Rett syndrome the observed loss of mitochondrial coupling would be expected to considerably reduce the capability of mitochondria to produce ATP via oxidative phosphorylation, thereby depleting cellular ATP levels. Glycolysis has been shown to be inhibited by ATP via inhibition of the phosphofructokinase-1 and pyruvate kinase enzymes (Larsson et al., 2000). Therefore a reduction in ATP levels due to mitochondrial inefficiency could result in the disinhibition of glycolysis and thereby increase levels of pyruvate and lactate.

Rett syndrome sufferers can present a number of common symptoms including loss of hand skills, hand stereotypies, loss of speech, social withdrawal, breathing difficulties, muscle wastage (Williamson & Christodoulou, 2006). Whilst it is difficult to define a direct link between mitochondrial dysfunction and this array of clinical symptoms, it is however possible to envisage that mitochondrial inefficiency could result in defects that manifest themselves on a cellular level thereby resulting in the acquisition of clinical symptoms.
6.17: Oxidative Stress and Rett Syndrome:

A role for oxidative stress in the production of the Rett Syndrome phenotype has been suggested. Erythrocyte SOD activity has been found to be reduced and plasma MDA (a marker of lipid peroxidation) concentrations increased although glutathione reductase, glutathione peroxidase and catalase activities were unchanged in erythrocytes (Sierra et al., 2001). Additionally lowered concentrations of ascorbate and glutathione have been identified in the postmortem brain of a Rett patient (Sofic et al., 1987), and was proposed as a mechanism which would lead to oxidative damage and neurological illness. As oxidative modification of MeCP2 has been shown to reduce its activity by at least 10-fold (Valinluck et al., 2004), a self-perpetuating model could be postulated whereby mutation of MECP2 leads to its reduced activity, leading to increased levels of oxidative stress and thereby further inhibiting MeCP2 function. Whilst study of the Mecp2-null mouse model of Rett syndrome cannot reveal whether this is the case, further experiments to determine if the loss of Mecp2 results in increased levels increased free radical production or compromised antioxidant defense could be enlightening.

The abnormalities detected in mitochondrial respiration give no clear indication that free radical production by the respiratory chain could be upregulated. Whilst there is increased respiratory capacity through Complex III, the mechanism underlying this increase in capacity is unknown making it difficult to speculate on whether the free radical generating property of Complex III will increase. The bifurcation of the electron transport pathway within the Q-cycle suggests that a linear backup of the respiratory chain to the antimycin blockade point when Complex III is operating submaximally will not occur. However a model can be proposed where, under coupled conditions, the entry of the second electron into the Q cycle is delayed when Complex III is upregulated, thereby stabilizing the ubisemiquinone and increasing free radical production. It is, however, unlikely that free radical production by the respiratory chain under physiological conditions will be increased in Mecp2-null mice due to the severe uncoupling effect observed.
Chapter 7: Conclusion
This thesis has reported evidence of mitochondrial abnormalities in the Edinburgh PrP-null mouse. Morphometric analysis of mitochondria isolated from brain tissue revealed that mitochondrial size and total cristae area were increased, whilst mitochondrial cristae density was decreased in PrP-null mice. The respiratory capacity of isolated mitochondria when metabolising Complex I substrates was found to be increased, and the capacity of Complex I to generate superoxide radicals was also increased in PrP-null mice. Previous studies and results presented in this thesis have indicated that PrP-null mice suffer oxidative stress via reduced antioxidant protection. The results presented in this thesis indicate that increased superoxide production by mitochondria may also be a contributory factor towards producing a state of oxidative stress. In addition neurons of the PrP-null mouse hippocampus CA1 region have been demonstrated to be more susceptible to degeneration under pro-oxidative conditions and an electrophysiological phenotype has been described in PrP-null mice whereby synaptic efficacy is reduced whilst field potential halfwidth is increased.

A variety of functions have been proposed for the endogenous prion protein, PrP\(^C\). Whilst it is difficult to reconcile how the loss of many of these putative functions could result in the abnormalities in PrP-null mice reported in this thesis, a model can be proposed whereby the loss of a SOD/antioxidant signaling function of PrP\(^C\) leads to the production of the observed phenotype.

Previous reports have suggested that PrP\(^C\) is a SOD enzyme and has roles in regulating antioxidant defenses, which agrees with the abnormalities in SOD activity reported within Chapter 4 of this thesis. The compromised antioxidant defense of PrP-null mice may then sensitize the neurons to oxidative insults, especially in regions where PrP\(^C\) expression is normally high such as the CA1 region of the hippocampus, as observed in Chapter 3 of this thesis. The fact that the phenotype was only observed under pro-oxidative conditions strongly implicates the involvement of oxidative stress in the production of this phenotype.
PrP-null mice may suffer from oxidative stress as a result of reduced SOD activity and reduced antioxidant protection from other antioxidant species. An increased level of oxidative stress may cause oxidative modification of the electron transport chain as has been previously reported elsewhere. As Complex I is inhibited by oxidative stress, it is possible that a compensatory increase in Complex I expression may occur in PrP-null mice. This would provide an explanation as to why Complex I activity and superoxide production from Complex I have been seen to be increased in PrP-null mice, as reported in Chapters 4 and 5 respectively.

It is difficult to propose a precise mechanism which would alter mitochondrial morphology due to the loss of PrP\textsuperscript{C}, as causes of morphological abnormalities in mitochondria are not well understood. However there are striking similarities between the mitochondrial morphological abnormalities reported in Chapter 4 and those that have been reported elsewhere in studies of other neurodegenerative conditions in which oxidative stress is thought to participate. It is therefore quite possible that increased levels of oxidative stress following the loss of PrP\textsuperscript{C} are responsible for the mitochondrial morphological abnormalities observed, and are a ubiquitous feature of oxidatively damaged mitochondria.

This thesis has also reported evidence of an electrophysiological phenotype in PrP-null mice which was variable between experimenters. The reduction in synaptic efficacy and increase in field potential halfwidth (presumed to indicate decreased GABAergic neurotransmission) in the PrP-null hippocampus may occur due to increased neuronal damage/death from oxidative stress during preparation of acute brain slices. The variability of results between experimenters suggests that a stressor encountered during slice preparation differentially affects the genotypes. This observation may have wider implications in relation to the variability reported in published PrP-null electrophysiology.

A summary diagram of this model is shown in Figure 7.1.
Figure 7.1: Summary of a proposed model to relate the results observed in PrP-null mice to each other.

Various components of this model could be tested experimentally. The involvement of oxidative stress in producing the variability in electrophysiological results between experimenters could be tested in acute slices by using different dissection techniques. This however would be extremely difficult to carry out in a quantitative fashion. A more practical alternative would be to study the electrophysiology of organotypic cell cultures, thereby permitting the study of neurons exposed to differing pro-oxidative environments. In addition the study of organotypic cell cultures could be expanded to test for other mitochondrial defects in PrP-null cultures, for example alterations in mitochondrial calcium buffering and mitochondrial membrane potential may be studied using fluorescent dyes.

Future experiments could also investigate the precise nature of the change in the respiratory chain. Whilst current evidence suggests that only a single subunit of
Complex I is upregulated in PrP-null mice (NADH B14.5b), re-visiting gene expression studies of PrP-null mice would be useful to confirm this. In addition it may be possible to determine whether the upregulation of this single subunit of Complex I is responsible for the observed changes in respiration rates and superoxide generation rates. This could be achieved by engineering and studying a cell line over-expressing NADH B14.5b in a similar way to that described for Uqerc1 in Chapter 6.

Whilst this thesis has reported that Complex I has an increased capacity to generate superoxide in PrP-null mouse brain, it was necessary to carry this study out in SMPs with the respiratory chain inhibited in order to prevent antioxidants interfering with the assay. It would be useful to determine whether increased superoxide production occurs in mitochondria under physiological conditions. Whilst none of the currently available spin traps could out-compete mitochondrial antioxidants, the development of better traps in the future may allow this line of enquiry to be pursued. The best alternative strategy given the currently available technology may be to repeat the study of superoxide production in SMPs using EPR, but instead titrating the respiratory chain inhibitors to produce pseudo-coupled SMPs.

This thesis has also reported alterations of the activity of the Electron Transport Chain, but not of the morphology, of mitochondria isolated from MeCP2-null mouse brain (a mouse model of Rett Syndrome). The alterations in the electron transport chain observed included an increase in respiratory capacity through Complex III and a loss of mitochondrial efficiency. This second finding has resonance with some of the symptoms of Rett Syndrome in humans. These mitochondrial abnormalities were only detectable in mice that had acquired overt clinical symptoms, suggesting that mitochondrial dysfunction may be causative towards symptom acquisition in this mouse model. The finding that the respiratory capacity through Complex III is increased in a cell-line overexpressing the Complex III subunit Uqerc1, suggests that Uqerc1 overexpression causes part of the observed mitochondrial phenotype. It would therefore
be of interest to determine whether overexpression of this subunit could produce Rett-like symptoms in a mouse model.

Whilst mitochondrial abnormalities were detected in both the PrP-null and the MeCP2-null mice, the mitochondrial phenotypes observed were clearly distinct from one another. The different mitochondrial abnormalities detected in PrP-null and symptomatic MeCP2-null mice may be one factor underlying the differential pathogeneses of Transmissible Spongiform Encephalopathies and Rett Syndrome respectively.
Reference List


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Appendix 1: Acquisition of accurate readings from oxygen electrode and EPR spectrometer

A1.1: Oxygen Electrode:

Whilst the Clark Oxygen Electrode is a relatively simple piece of equipment, a number of steps were taken to ensure the data acquired was of high quality.

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Figure A1.1.1: Schematic diagram showing the essential features of the oxygen electrode used for respiration measurements, diagram is not drawn to scale.
• Before setting up the electrode, the platinum cathode and silver anode were polished to reduce noise in the recording. Tissue paper soaked in 3M KCl was placed over the electrodes to act as an electrolyte. A square of oxygen-permeable Teflon membrane was placed over this and was locked in position by the body of the oxygen electrode chamber. Care was taken to ensure that there were no leaks from the chamber body into the electrolyte solution as this would have resulted in an unstable or open circuit.

• The cathode was polarized to approximately -0.6V relative to the anode, however the electrode took about 24 hours to fully polarize. As this affected the sensitivity of the electrode, experiments typically were not carried out until 48 hours after setting up the electrode at which point it was fully polarized and hence the electrode sensitivity was stabilised.

• A large reservoir of water was maintained in the water bath which fed the water jacket as this minimized fluctuations in temperature which could affect the recording.

• As the reaction at the cathode consumes oxygen it was necessary to ensure that the solution in the chamber was constantly stirred so that a shell of low oxygen concentration did not build up around the cathode.

• Care was taken to ensure that no bubbles remained in the chamber once recording commenced since the presence of air-bubbles tends to buffer the oxygen levels within the solution.

• To minimize diffusion of oxygen back into solution from the atmosphere, care was taken to ensure that the fluid level within the chamber was within the thin injection port tube when readings were taken. To achieve this, the height of the cap was adjusted using the locking ring.

_A1.2: EPR spectrometer:_
As the MS100 EPR spectrometer has a detection area covering a radius of approximately 10mm, it was necessary to ensure that samples were prepared in a way which would minimise inter-sample variability. The 120mm long holding tube was positioned within
the clamping cone so that 10mm of the tube was visible above the cone. When in this position the holding tube extends 110mm below the top of the clamping cone and the point of peak microwave intensity is 20mm from the base of the holding tube. As the sensitive region within the spectrometer extends to an approximate radius of 10mm from this point, samples were placed in capillaries ensuring that the base of the solution was within 10mm of the end of the capillary. The depth of sealant compound and the airspace of prepared capillaries was less than 10mm sum and capillaries were filled up to the 50μl calibration line. These steps ensured that the region of the capillary within the sensitive area was completely filled with the sample, thereby reducing one potential source of error in EPR recording.

Figure A1.2.1: Diagrammatic representation of the features essential for suitable positioning of EPR samples within MS100 EPR spectrometer (Magnettech). Figure is not drawn to scale.
Appendix 2: Analysis of EPR spectra

A2.1: Peak to Peak versus Area Under the Curve:
As the peak width of tempone EPR spectra remains constant with tempone concentration (e.g. for spectra in Figure 5.12.1 from 1 to 10μM, peak width = 2.11 G ± 0.01 SEM), it is possible to assess EPR signal intensity as either the peak to peak amplitude of the waveform or by the 2nd integral (AUC) of the spectrum (note that the EPR spectrum is recorded as the derivative of the absorption spectrum). To determine the correlation between these two methods of analysing EPR spectra, a variety of tempone samples were analysed using both methods and are plotted in Figure A2.1.1.

![Scatter plot demonstrating the relationship between the signal amplitude and the AUC (area under the curve) for tempone EPR spectra.](image)

Figure A.2.1.1: Scatter plot demonstrating the relationship between the signal amplitude and the AUC (area under the curve) for tempone EPR spectra. n=78, r² = 0.998.

The data in Figure A2.1.1 demonstrates a high degree of correlation between the peak to peak amplitude and 2nd integer methods of assessing EPR signal intensity. However both analysis techniques possess inherent limitations. Signal noise can contaminate peak to peak amplitude measures, making assessment of small EPR signals problematical. This can be overcome by averaging a number of sweeps to even out the noise levels but this reduces the temporal resolution of the experiment. A balance between obtaining clean spectra and the ability to acquire multiple spectra rapidly is therefore necessary when using this analysis technique. AUC measurements are less
affected by signal noise, but require a correction for the baseline drift which underlies EPR signals. This drift correction has to be carried out manually and involves a degree of subjectivity. It can also be difficult achieve accurately using the available analysis software. This correction becomes of more important when EPR signals are small when any underlying background drift becomes more significant (see Figure A2.1.2).

Figure A2.1.2: Panel A: Original spectra from tempone solutions recorded using parameters stated in methods Section. Top trace shows spectra from 20μM tempone at gain of 50. Bottom trace shows spectra from 2μM tempone at a gain of 500. Arrows on the left represent the measurement of the peak to peak amplitude measure of EPR spectrum intensity. Panel B shows the first integral of the spectra shown in panel A.

The data in Figure A.2.1.2 panel A shows an original spectrum from 20μM tempone (top), and a spectrum from 2μM tempone (bottom) acquired using a higher gain setting so that it matches the size of the top spectrum. Although the signal to noise ratio is significantly higher in the bottom spectrum, it has no great effect upon the peak to peak measure of spectrum intensity. Panel B shows the first integrals (i.e. absorption spectra) of the EPR spectra shown in Panel A. The absorption spectrum from the 20μM sample shows three distinct peaks of equal magnitude and low levels of background drift. However the 2μM sample shows considerable background drift which must be corrected.
for prior to measurement of the AUC. This considerably reduces the reliability of measuring tempone spectra using the AUC analysis protocol when EPR spectra are small due to the considerable influence of baseline fitting on the final measurement. As the peak to peak amplitude measure is unaffected by inaccurate baseline fitting by subjectivity during analysis and because noise interference can be minimised by performing multiple sweeps, the peak to peak analysis procedure will be used to analyse all data reported in subsequent Sections.

A2.2: Waveform Subtraction procedure:
To analyse spectra relative to their controls, a waveform subtraction procedure was carried out, as illustrated in Figure A2.2.1.

![Waveform Subtractions](image)

**Figure A2.2.1:** Original spectra for control and experimental conditions (left) and waveform subtracted spectra (right). The blue traces show an experimental sample that has produced a spectra of greater magnitude than control thereby producing a positive waveform subtracted spectra, whereas the red traces show an experimental sample that has produced a spectra of lesser magnitude than control leading to the production of an inverse spectra following waveform subtraction. The production of inverse spectra has no influence on the peak to peak measure of spectra intensity apart from the need to denote these spectrum intensities as negative.
APPENDIX 3: Tempone-H auto-oxidation:

A3.1: Metal contamination of buffer solutions:
Tempone-H in solution is gradually converted into tempone in a process known as auto-oxidation. A scheme by which auto-oxidation occurs has been suggested by Dikalov et al (Dikalov et al., 1999) which involves the cyclical reduction and oxidation of redox active metals such as copper or iron:

\[ M^{n+j+} + \text{Tempone-H} \rightarrow \text{Tempone} + M^{n^+} \]
\[ M^{n^+} + O_2 \rightarrow M^{n+j^+} + O_2^* \]

Where M is any redox active metal

![Graph showing the effects of copper ions upon the auto-oxidation rate of 1mM tempone-H. The change in tempone EPR signal was determined over a 30 minute period in the presence of 0, 0.5 and 1 μM copper sulphate, and in the presence of 1μM copper sulphate and 100μM EDTA. All data was background subtracted for the auto-oxidation rate in the absence of exogenous copper. n=3, error bars represent SEMs.]

Figure A3.1.1: The effects of copper ions upon the auto-oxidation rate of 1mM tempone-H. The change in tempone EPR signal was determined over a 30 minute period in the presence of 0, 0.5 and 1 μM copper sulphate, and in the presence of 1μM copper sulphate and 100μM EDTA. All data was background subtracted for the auto-oxidation rate in the absence of exogenous copper. n=3, error bars represent SEMs.

The data in Figure A3.1.1 supports the theory that free transition metal ions can increase the auto-oxidation rate of tempone-H. This increase in auto-oxidation rate is prevented
by the addition of 100µM EDTA. This data underlines the importance of controlling metal ion concentrations in EPR samples as small changes in free redox active metal concentrations may considerably alter the auto-oxidation rate. It is therefore possible that tempone EPR signals could be erroneously reported as superoxide/peroxynitrite mediated when they are actually due to an increase auto-oxidation resulting from metal contamination (Dikalov et al., 1999).

This scheme of auto-oxidation suggests that transition metal contaminants present in biological media can act catalytically to oxidize tempone-H to tempone, hence producing a spin signal. The oxidation of tempone-H would occur both via direct reaction of oxidized metal with tempone-H, and via tempone-H oxidation by superoxide produced during reduction of molecular oxygen by the metal.

As spin-trap auto-oxidation produces a background signal against which any biological radical production would have to be measured, it is desirable to minimize trace metal contamination of buffer solutions. The ion exchange resin chelex-100 (Sigma) was investigated as a method by which to remove redox-active transition metals from buffer and reagent solutions.

The effectiveness of chelex resin treatment in reducing tempone-H oxidation rates was investigated using 1mM tempone-H in phosphate buffered potassium salt buffer pH 7.4. The data in Figure A3.1.2 Panel A demonstrates that treatment of buffers with chelex resin reduced the rate of auto-oxidation of tempone-H by approximately 10-fold. This is due to the chelex resin exchanging divalent cations for sodium ions, thereby reducing the concentrations of redox active metals and lowering auto-oxidation rates.
Figure A3.1.2: Panel A: Comparison of the rates of tempone EPR signal development due to auto-oxidation of 1mM of tempone-H in phosphate buffered potassium salt pH 7.4 (filled circles), and in the same buffer treated with chelex resin for 48 hours and pH adjusted to 7.4 (open circles). Linear regressions applied to all data under untreated (blue line) and chelex treated (red line) conditions are shown, error bars represent SEM. Statistics were performed on linear regression gradients for each experiment, for untreated buffer mean gradient was 83.3nMmin⁻¹ ± 4.6, for chelex treated buffer mean gradient was 8.3nMmin⁻¹ ± 1.7. n = 5 for each condition. P < 0.001, Student's unpaired t-test. Panel B: Auto-oxidation rates of 1mM tempone-H dissolved in phosphate buffered saline pH 7.4 treated with either new chelex (filled circles) or regenerated chelex (open circles). Linear regressions applied to all data within new chelex (blue line) and regenerated chelex (red line) treated buffer datasets. Error bars represent SEM, n = 3.
To achieve a low background auto-oxidation rate, all subsequent experiments employed buffers prepared from Millipore filtered water and treated with 5g of chelex resin per 100ml for a minimum of 6 hours.

It is also worth noting that the linear regressions in Figure A3.1.2 do not pass precisely through the origin but cut the y-axis above 0. This occurs because tempone-H auto-oxidation begins when tempone-H is dissolved into solution, which was not synonymous with time zero in the experiment.

The repeatability of the chelex treatment protocol was assessed to confirm its reliability. To this end three separate batches of buffer were treated with chelex resin, and the rate of EPR signal development monitored over 90 minutes. Auto-oxidation rates of 1mM tempone-H in 3 separately prepared batches of chelexed buffer were similar to within 20% of each other. However as tempone-H auto-oxidation rates are dependent upon both transition metal contamination and tempone-H concentration, it was necessary to perform auto-oxidation controls in every subsequent experiment to account for these variables.

It has been reported that Chelex resin can be regenerated by treatment with acid followed by alkali (BioRad Chelex 100 and Chelex 20 chelating ion exchange resin instruction manual Lit200 Rev B). However the data in Figure A3.1.2 panel B shows that when regenerated chelex was used to treat a phosphate buffered salt solution, the rate of auto-oxidation was significantly higher than when new chelex was used (new chelex treated buffer 5.8nMmin⁻¹ n = 3, regenerated chelex treated buffer 10.9nMmin⁻¹). As regenerated chelex appears to be less effective than new chelex, all subsequent experiments utilized fresh chelex resin.

A3.2: Auto-oxidation and enzyme stocks:
Enzymes can be useful tools for assigning EPR signals to specific radical species. However enzyme preparations are only useful if they affect the EPR spectra by their
catalytic effects, rather than by altering EPR signal evolution due to metal contamination. The enzyme Superoxide Dismutase (SOD), which catalyses the conversion of superoxide to hydrogen peroxide and oxygen, is one such useful enzymatic tool as it can help verify whether an EPR signal is superoxide-dependent.

An initial experiment using copper zinc superoxide dismutase (CuZn SOD) from bovine erythrocytes demonstrated that auto-oxidation of tempone-H increased considerably in a linear fashion with SOD concentration over the concentration range of 10 to 500U/ml. This finding was somewhat counterintuitive as the enzymatic activity of SOD would be expected to partially reduce the auto-oxidation rate. However, whilst chelex treatment of buffer solution removes one potential source of metal contamination, the introduction of metal contaminants in enzyme preparations is unaffected by the chelex treatment. It is therefore likely that metal contamination of the SOD stock was responsible for the observed effect. The feasibility of using dialysis to clean enzyme stocks prior to their use in EPR was therefore investigated.

Figure A3.2.1 demonstrates that the addition of 200U/ml of untreated CuZn SOD greatly increased tempone-H auto-oxidation levels relative to control. Initial attempts to clean enzyme stocks utilized cellulose membranes of 3,000 MWCO to retain the enzyme protein whilst allowing small molecules such as metal ions to be filtered out. However, initial dialysis attempts using cellulose membranes resulted in a considerable increase in the auto-oxidation rate of tempone-H, presumably due to release of redox-active metals from the cellulose dialysis membrane. Treatment of the cellulose membranes to remove metals from them using established protocols reduced the auto-oxidation rate to below control levels. These results indicate that dialysis using cleaned cellulose membranes efficiently removes metal contamination from enzyme stock solutions, thus permitting their use in EPR experiments. All enzyme solutions used here were therefore dialysed prior to use in EPR experiments.
Figure A3.2.1: Effects of dialysis upon auto-oxidation rate of tempone-H with 200U/ml SOD. Control samples (filled circles) consisted of 1mM tempone-H in chelex treated phosphate buffered saline and were supplemented with 200U/ml undialysed CuZn SOD from bovine erythrocytes (open circles), 200U/ml CuZn SOD dialysed against chelex treated buffer using cellulose membranes (filled triangles) or addition of 200U/ml SOD which was dialysed using bicarbonate/EDTA treated cellulose membranes (open triangles). Linear regressions were applied to all data within control (blue line), undialysed SOD (red line), SOD dialysed within uncleane...
Appendix 4: Reduction of tempone by biological antioxidants

A4.1: Ascorbate

A potential problem associated with the use of tempone-H as a spin trap is that tempone has been reported to be subject to reduction by some anti-oxidants (Dikalov et al., 1997b). Such anti-oxidant activity would result in a loss of tempone and hence underestimation of superoxide production. As biological systems posses a myriad of anti-oxidant defences, the effects of some anti-oxidants upon tempone EPR signals were investigated. The antioxidants investigated here were those considered important within the mitochondria, as the final aim was to measure superoxide production by the respiratory chain. For the purposes of these experiments 1 mM tempone was used to assess the impact antioxidants have upon tempone EPR signals.

Figure A4.1.1 panel A shows that addition of ascorbate to 1 mM tempone results in a concentration dependent loss of tempone spin signal which develops within a period of approximately 30 minutes. The slope of the regression in panel B demonstrates that stoichiometry of this reaction is approximately a loss of 1.6 moles of tempone per mole of ascorbate, possibly indicating that ascorbate is being oxidized to SDA which may dismutate resulting in the formation of additional ascorbate. These results demonstrate that ascorbate has a potent antioxidant effect upon tempone.
Figure A4.1.1: Panel A: Time-course of evolution of signal inhibition following the addition of ascorbate at varying concentrations to a solution of 1mM tempone. EPR signal inhibition is evident even 1 minute after the addition of the antioxidant, and is over 75% complete within 15 minutes. Panel B: The magnitude of signal inhibition produced over 120 minutes at 30°C by the addition of set amounts of ascorbate to 1mM 4-oxo tempone. The relationship between ascorbate concentration and signal inhibition is linear ($r^2=0.987$) in the range shown, above the 500µM ascorbate concentration linearity was lost due tempone becoming the limiting factor. N=3, error bars are SEMs.
A4.2: Glutathione

Figure A4.2.1: Effects of reduced glutathione upon tempone EPR signals. Panel A: Time-course of 1mM tempone EPR signal inhibition in the presence of various concentrations of reduced glutathione. Maximal signal inhibition was reached after 1 hour incubation at 30°C. Panel B: EPR Signal inhibition achieved following 2 hours of incubation of GSH with 1mM tempone at 30°C plotted against GSH concentration. A linear relationship was observed between GSH concentration and EPR signal inhibition ($r^2 = 0.981$).
The data in Figure A4.2.1 panel A demonstrates that addition of reduced glutathione to a tempone solution causes a rapid reduction in the tempone EPR signal from the solution. The likely explanation for this is that the reduced glutathione reduces the tempone, converting it to an EPR silent species. The slope of the linear regression in panel B indicates that the reaction appears to occur with a 1:1 stoichiometry i.e. 1 mole of tempone reduced for every mole of glutathione present. As reduced glutathione is a common cellular antioxidant, and is thought to be present at up to millimolar levels in the mitochondria (Halliwell & Gutteridge, 1999), the data suggests glutathione could potentially significantly interfere with any attempt to measure superoxide production by mitochondria.

The data in Figure A4.2.1 panel A shows an apparent lag prior to the maximal rate of glutathione mediated tempone EPR signal loss. The possibility of reaction temperature producing this phenomenon was studied.

![Figure A4.2.2: Plot of EPR signal inhibition against time when 1mM tempone was incubated with 500uM GSH at different temperatures. n = 1 for all points](image)

Figure A4.2.2: Plot of EPR signal inhibition against time when 1mM tempone was incubated with 500uM GSH at different temperatures. n = 1 for all points
The data shown in Figure A4.2.2 suggests that at temperatures lower than 37°C the lag before the maximal rate of tempone reduction was considerably increased, to such an extent that at 25°C it was between 30 and 60 minutes before any significant reduction in tempone EPR signal was observed. To prevent the variable time of onset of this lag from affecting results from future experiments, assessment of antioxidant effects of biological preparations was carried out at 37°C.

In order to improve the resolution of these antioxidant reactions, the feasibility of using low tempone concentrations to detect small quantities of antioxidants was investigated. However when tempone concentrations were low (<10μM) no EPR signal inhibition was observed upon the addition of GSH up to concentration of 50μM (data not shown). This suggests that interaction between tempone and glutathione is unfavourable when tempone concentrations are low, and thereby the assay of antioxidants using low tempone concentrations is unfeasible.

Tempone concentrations remain relatively low during tempone-H spin trapping experiments and it is therefore debatable whether the activity of low molecular weight antioxidant such as ascorbate or GSH would have any impact upon measurement of superoxide generation under these conditions. However it is difficult to assess how these reactions will proceed in biological preparations as a vast number of other factors may alter the antioxidant effect. Possibilities for modification of the reaction include the participation of enzymes (such as glutathione peroxidase) and interaction with other antioxidant systems.

A4.3: CuZn SOD and ferricytochrome c:

The data in Figure A4.3.1 demonstrates that neither SOD nor ferricytochrome c at high concentrations are able affect tempone EPR spin signals. This indicates that SOD cannot inhibit tempone EPR signals following tempone formation. However it can catalytically remove superoxide thereby reducing tempone-H auto-oxidation. The inability of ferricytochrome c, a mitochondrial component, to reduce tempone EPR
signals indicates that it cannot be responsible for any tempone EPR signal observed when biological preparations are used.

Figure A4.3.1: Potential interactions of other antioxidants with tempone. Panel A: EPR signals from 500µM tempone alone (black bars) and in the presence of 10,000 U/ml dialysed CuZn SOD (grey bars) incubated at 37°C. N = 3, error bars represent SEMs. Panel B: EPR signals from 500µM tempone alone (black bars) and in the presence of 500µM ferricytochrome c (grey bars). N = 3, error bars represent SEMs.
Appendix 5: Alternative spin traps

A5.1: DMPO:

The intrinsic stability of tempone in solution coupled to its susceptibility to reduction by antioxidants makes it difficult to measure rates of superoxide production rather than antioxidant effects. Therefore other spin traps were assessed as potential candidates for assessing superoxide production by mitochondria.

5, 5-Dimethyl-1-pyrroline-N-oxide (DMPO) has long been considered a spin trap useful for the assessment of superoxide production. DMPO spin trap superoxide adducts are less stable than tempone and do not accumulate, and DMPO is subject to lower levels of auto-oxidation. DMPO can therefore be used to provide a ‘snap-shot’ of superoxide production rates, which will take into account antioxidant effects.

However DMPO superoxide adduct spectra acquired using the MS100 spectrometer had a very poor signal to noise ratio even when a concentration of 160mM DMPO was utilized (Figure A.5.1.1 panel A). The high levels of noise encountered in DMPO spectra made the peak to peak analysis measurement unreliable. Additionally the absorption spectrum (Figure A5.1.1 panel B) showed extreme levels of background drift which made AUC analysis of spectra unreliable. Therefore using our experimental setup it was not possible to obtain reliable quantitative data when using DMPO as a spin trap for superoxide radicals.
Figure A5.1.1: Panel A: Spectrum of DMPO superoxide radical adduct exhibiting 4 peaks in a 1:2:2:1 ratio. Panel B: The EPR absorption spectrum calculated as the first integral of the EPR spectrum.

A5.2: CP-H:

A nitrooxide spin trap CP-H similar to tempone-H was investigated as it is reported to exhibit less susceptibility to reduction by antioxidants (Dikalov et al., 1997b). Following oxidation of CP-H by superoxide, the radical 3-CP is formed which has a triplet EPR signal similar to that of tempone.

As shown in Figure A5.2.1 panel A 3-CP exhibits similar triplet EPR signals to tempone, although the spectra tend to exhibit a lower amplitude and greater width than equal concentrations of tempone when using identical EPR spectrometer settings. This results in EPR intensities, when measured as peak to peak amplitude, appearing lower than equivalent concentrations of tempone, although when the AUC analysis protocol is employed EPR signal strength from equal concentrations of tempone and 3-CP are similar. Panel B shows that there is no obvious loss of 3-CP EPR signal within a ninety minute time period when 3-CP was dissolved in PBKCI pH7.4. This demonstrates that, like tempone, 3-CP is stable in solution. Panel C demonstrates that there is a linear
relationship between 3-CP concentration and EPR signal intensity within the concentration range of 0 to 100\(\mu\)M 3-CP.

**Figure A5.2.1:** Panel A: EPR spectra of varying concentrations of 3-CP in phosphate buffered potassium salt pH7.4, incubated at 30\(\degree\)C for 10 minutes (need to put in concentrations) Panel B: The effect of time upon EPR signal intensity at different concentrations of 3-CP. Error bars represent SEM, linear regressions applied to all data at each tempone concentration, \(n = 3\). Panel C: The relationship between tempone concentration and ESR signal amplitude following 10 minutes incubation at 30\(\degree\)C. Error bars represent SEM, \(n = 3\).
Figure A5.2.2: Comparison of the EPR signal intensities obtained from varying concentrations of 3-CP (filled circles) and from CP-H treated with 20mM KO$_2$ for 4 hours (open circles). 3-CP/CP-H was dissolved in a buffer composed of 50mM NaOH and 1mM EDTA disodium salt. $n=3$. Linear regressions were applied to all data for 3-CP (blue line) and KO$_2$ treated CP-H (red line).

To confirm that the spin trap CP-H could be converted to 3-CP thus producing a spin signature, known concentrations of CP-H were treated with 20mM KO$_2$ 4 hours prior to EPR signal detection. No significant differences were detected between the signals obtained from 3-CP and CP-H as shown in Figure A5.2.2. This demonstrates that CP-H can be converted to a compound which shows a similar spin signature to 3-CP, and as the signals are proportional to each other at a ratio of 1:1, the stoichiometry of the reaction is as predicted with one mole of CP-H converted to 1 mole of 3-CP:

$$\text{CP-H} + \text{O}_2^{\cdot} \rightarrow \text{3-CP} + \text{H}_2\text{O}_2$$
Figure A5.2.3: Graph showing the time-course of the evolution of an EPR signal when 1mM CP-H is incubated with an X/XO superoxide generation system at 30°C, and controls demonstrating the superoxide dependence of signal evolution. Xanthine concentration 0.5mM, xanthine oxidase concentration 0.001U/ml, SOD concentration 50U/ml and catalase concentration 100U/ml. Error bars represent SEM, n = 3.

Figure A5.2.3 demonstrates that CP-H can be used as a spin trapping agent for the superoxide produced by a xanthine/xanthine oxidase couple. Firstly it was proved that the complete enzyme/substrate couple resulted in considerably greater production of 3-CP than would be expected for auto-oxidation of CP-H as shown in the controls. The signal was inhibited by the addition of SOD, but not by catalase, demonstrating that superoxide was responsible for the increased 3-CP spin signals. However the data in Figure A5.2.3 also suggests that the auto-oxidation of CP-H was considerably higher than that of tempone-H. To determine if this was the case the auto-oxidation rate of 1mM CP-H was compared to that of 1mM tempone-H within a single batch of chelex treated phosphate buffered saline.
Figure A5.2.4: Comparison of the rates of auto-oxidation of 1mM Tempone-H (filled circles) and 1mM CP-H (open circles) in chelex treated phosphate buffer salt solution (pH7.4) incubated at 30°C. Linear regressions were applied to all data for tempone-H (blue line) and CP-H (red line) auto-oxidation. Error bars represent SEMs, n=3.

Figure A5.2.4 demonstrates that the auto-oxidation rate of 1mM CP-H is approximately 3 times greater than that of tempone-H. This may either be due to a greater susceptibility of CP-H to auto-oxidize, or may be caused by a higher level of redox-active metal contamination in CP-H than in tempone-H. This increased auto-oxidation rate is detrimental to the use of CP-H as a spin-trap as it increases the rate of background signal evolution against which any biologically derived EPR signal would have to be measured against. Despite possessing an increased auto-oxidation relative to tempone-H, the use of CP-H could be justified if it could be shown to be considerably more antioxidant resistant.
Figure A5.2.5: The effects of ascorbate upon 3-CP EPR signals. Panel A: Time-course of evolution of signal inhibition following the addition of varying concentration of ascorbate to 1mM 3-CP. Panel B: Relationship between ascorbate concentration and the EPR signal inhibition following 120 minutes incubation at 37 °C. The relationship between the amount of ascorbate added and the magnitude of EPR signal inhibition is linear over the range investigated ($r^2 = 0.985$). n=3, SEM error bars.
Figure A5.2.6: Effects of reduced glutathione upon 3-CP EPR signals. Panel A: Time-course of 1mM 3-CP EPR signal inhibition in the presence of various concentrations of reduced glutathione, incubated at 37 °C. Panel B: Magnitude of EPR Signal inhibition achieved following 2 hours of incubation of GSH with 1mM 3-CP at 37 °C plotted against GSH concentration. A linear relationship ($r^2 = 0.942$) was observed between GSH concentration and EPR signal inhibition.
The data in Figure A5.2.5 demonstrates that the reaction between 3-CP and ascorbate appears not to have reached completion following 2 hours of incubation, and following this period only small percentage of the ascorbate is oxidized (in the case of 500μM ascorbate 2.5% of the 3-CP EPR signal is lost). Despite the reaction appearing not to have reached completion, the relationship between ascorbate concentration and 3-CP EPR signal inhibition is linear when a single time-point is considered. When compared to the reaction of tempone with ascorbate (see Figure A4.1.1), the reaction of ascorbate with 3-CP at a considerably reduced rate which supports the theory that 3-CP is more resistant to antioxidants than tempone.

The data in Figure A5.2.6 demonstrates that reduced glutathione can have a potent effect upon 3-CP EPR signals. Figure A5.2.6 panel B demonstrates that following 3 hours incubation at 37°C, the stoichiometry of the reaction is approximately 1 mole of GSH reduces 0.9moles 3-CP. Interestingly a considerably lag was observed of between 60 and 90 minutes was routinely observed before any inhibition of 3-CP EPR signal was detectable. However the applicability of the use of this phenomenon in the analysis of biological preparations is uncertain, as the reaction of GSH with 3-CP may be subject to modification by other components of the biological system.

Whilst 3-CP is less susceptible to reduction by ascorbate, it’s susceptibility to reduction by GSH is similar to that of tempone. In addition the auto-oxidation rate of CP-H was determined to be 3 times that of tempone-H. The considerably greater auto-oxidation rate of CP-H relative to tempone-H would result in larger background signals against which to measure superoxide production in biological samples. This deleterious feature of CP-H outweighs the benefit of 3-CP’s increased resistance to reduction by cellular antioxidants, and therefore tempone-H was deemed the better spin-trap for the purposes of this study.
Appendix 6:
Paper Published in Molecular and Cellular Biology

"Gene expression analysis exposes mitochondrial abnormalities in a mouse model of Rett Syndrome"

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Gene Expression Analysis Exposes Mitochondrial Abnormalities in a Mouse Model of Rett Syndrome

Skirmantas Kriaucionis,1 Andrew Paterson,2 John Curtis,2 Jacky Guy,1 Nikki MacLeod,2 and Adrian Bird1*

Wellcome Trust Centre for Cell Biology, University of Edinburgh, The King's Buildings, Edinburgh EH9 3JR, Scotland, United Kingdom,1 and Division of Biomedical Sciences, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, Scotland, United Kingdom2

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Rett syndrome (RTT) is a severe neurological disorder caused by mutations in the X-linked MECP2 gene, which encodes a methyl-CpG binding transcriptional repressor. Using the Mecp2-null mouse (an animal model for RTT) and differential display, we found that mice with neurological symptoms overexpress the nuclear gene for ubiquinol-cytochrome c reductase core protein 1 (Uqcrcl). Chromatin immunoprecipitation demonstrated that MeCP2 interacts with the Uqcrcl promoter. Uqcrcl encodes a subunit of mitochondrial respiratory complex III, and isolated mitochondria from the Mecp2-null brain showed elevated respiration rates associated with respiratory complex III and an overall reduction in coupling. A causal link between Uqcr1 gene overexpression and enhanced complex III activity was established in neuroblastoma cells. Our findings raise the possibility that mitochondrial dysfunction contributes to pathology of the Mecp2-null mouse and may contribute to the long-known resemblance between Rett syndrome and certain mitochondrial disorders.

Rett syndrome (RTT) is a profound neurological disorder that almost exclusively affects girls. Approximately 80% of patients possess a mutation in one copy of the X-linked MECP2 gene that is, except in very rare cases, absent in somatic DNA from the parents (1). It follows that MECP2 mutations are the underlying cause of RTT. Mice lacking an intact Meep2 gene have been generated in several laboratories (5, 10, 37). Without the Meep2 gene, mice are born, but they acquire neurological symptoms after about 6 weeks and die at about 10 weeks of age (5, 10). Females heterozygous for the disrupted Meep2 allele are apparently normal for several months, producing multiple litters, but later display symptoms similar to those of the Mecp2-null mice. An important difference is that their health does not progressively decline but remains stable for an apparently normal life span (10). These heterozygous animals are genetically more comparable to RTT patients, and their delayed-onset neurological symptoms and abnormal gait recall the human condition. The Mecp2-null mouse therefore provides an animal model for human RTT with the potential to shed light on its underlying molecular causes (19).

MeCP2 is a nuclear protein that binds preferentially to methylated sites in chromosomal DNA (22, 27, 29) and can function as a DNA methylation-dependent transcriptional repressor (27). Analyses of the Mecp2-null mouse therefore sought genes that might be overexpressed due to an absence of repression by MeCP2. Microarray analysis of brain RNA showed small alterations in the levels of multiple mRNAs when wild-type (wt) and Mecp2-null samples were compared (38). Although the effects were insignificant for individual genes, they became statistically significant when groups of affected genes were considered. Success in finding direct targets of MeCP2 was first achieved via a “candidate gene” approach (6, 24). One of four promoters of the gene for brain-derived neurotrophic factor (Bdnf) was found to bind MeCP2. This promoter is activated by artificial stimulation of cultured murine neurons. Remarkably, MeCP2 becomes phosphorylated upon stimulation and is lost from the promoter, thereby temporarily relieving MeCP2-mediated repression. Recent studies have additionally reported abnormal expression of the Dlx-5 and Dlx-6 (13), Ube3A and Gabrb3 (35), and Sgk1 and Fkbp5 (31) genes as a target genes for MeCP2.

In an attempt to detect additional MeCP2-regulated genes and assess their contributions to phenotype, we subjected mRNA from Mecp2-null mouse brains to global analysis of gene expression by using a variant of differential display (17). Populations of mRNAs from mutant and wild-type brains were visualized by amplifying cDNA subsets that were then displayed by gel electrophoresis. Reproducible differences in the intensities of the resulting bands indicate altered expression. Our analysis revealed that the gene encoding a component of the mitochondrial respiratory chain is misregulated in the mutants at a time when symptoms are just beginning. We hypothesized that the phenotype of these mice might involve mitochondrial abnormalities, and we therefore investigated mitochondrial respiration in Mecp2-null mouse brain by using an oxygen electrode. The results showed abnormal respiration in brain mitochondria from mutant symptomatic mice compared to wild-type controls. Blue native gel electrophoresis of respiratory complexes followed by enzymatic staining showed reduced activity in some components of the respiratory chain. Overexpression of the Uqcrcl gene in cultured cells induced physiological changes in mitochondria that resembled those seen in the Mecp2-null mouse brain. Our data therefore raise the possibility that some of the characteristics of the mouse
phenotype, and perhaps of human RTT, are mitochondrial in origin.

MATERIALS AND METHODS

RNA isolation. RNA was purified from whole brain using TRI reagent (Sigma) according to the manufacturer’s recommendations.

ADDER differential display. Pooled total RNA (three RNA pools, each consisting of three separate RNA preparations from nine brain samples in total) from MeCP2-null male mice (C57/black six) and the same number of control littermates was used as an input for ADDER (amplification of double-stranded cDNA and restriction fragments) analysis. ADDER was performed as described previously (17) with minor modifications. Briefly, pooled total RNA samples were used for cDNA synthesis, using a poly(A)-amplifying oligonucleotide with a biotin label at its 5' end. cDNA was then adsorbed on magnetic streptavidin beads and cut with Mbol (NEB) restriction endonuclease. The resulting DNA ends were ligated to oligonucleotide adaptors and released from beads by Acld (NEB) digestion. cDNA fragments were amplified and used as templates for differential display PCR using primers with different terminal nucleotides (Fig. 1a). Bands with different intensities were cut from the gel and eluted DNA was PCR amplified using primers with a specific terminal 3' dinucleotide pair. Reamplified DNA was cloned into a plasmid vector. Restriction analysis of 24 picked clones allowed identification of the misexpresed fragment.

Reverse transcription and real-time PCR analysis. The RNA pools were used for cDNA synthesis as described elsewhere (18). Real-time PCR analysis was performed using an iCycler real-time PCR machine (Bio-Rad). Four parallel reactions were carried out for each cDNA pool with IQ SYBR green Supermix (Bio-Rad) or homemade mix (0.5 μM SYBR green [Molecular Probes], 10 μM fluorescein (Sigma), 1× PCR buffer with MgCl2 [2 mM final concentration; Roche], and 200 μM deoxynucleotide triphosphates [Ambion] with 1 U FastStart Taq polymerase [Roche]). Results were always displayed relative to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA. Primers used for this analysis are shown in Table 1.

Chromatin immunoprecipitation. Three whole brains (from adult mice age 2 to 5 months) provided sufficient input material for six or seven immunoprecipitations. Pulsed-frozen brains were ground in liquid nitrogen, and the powder was poured into fixation solution (1% formaldehyde in phosphate-buffered saline). Fixation was continued for 15 min at room temperature and quenched with 0.125 M (final concentration) glycine solution. Pelleted cells were washed with phosphate-buffered saline and homogenized in a Dounce homogenizer. After centrifugation, cells were resuspended in 9 ml of cell lysis buffer (0.2% NP-40, 10 mM NaCl, 10 mM Tris-HCl [pH 8]), Complete [Roche] protease inhibitors). Lysate was triturated through a 25-gauge needle to remove clumps and incubated for 15 min with an additional 6 ml of lysis buffer. Nuclei were then harvested by spinning at 4,000 g for 5 min and resuspended in 3.6 ml of nucleus lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 1% sodium dodecyl sulfate [SDS], protease inhibitors). Nuclei were lysed for 10 min at room temperature and diluted with 2.2 ml of immunoprecipitation dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris HCl [pH 8], protease inhibitors). Chromatin was sonicated twice for 4 min with a Branson Sonifier 250 (duty cycle 60; output, 6) to obtain chromatin DNA fragments of about 200 bp on average. Chromatin was then cleared by centrifugation, diluted five times, precleared with protein A-Sepharose (Amersham), and subjected to overnight immunoprecipitation with rabbit polyclonal antibody 674 against MeCP2 (28) and rabbit polyclonal histone H3 dimethyl K9 antibody (Abcam). Antibody precipitates were bound to protein A-Sepharose for 1 h. Washes were performed once with TSE1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl [pH 8]), four times...
**TABLE 1. Primers used for real-time PCR analysis**

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| BC058513           | Snap70      | **Bisulfite DNA modification.** Bisulfite DNA modification was performed using standard procedures. Briefly, 1 μg of whole brain genomic DNA was digested with BamHI (NEB) restriction endonuclease, dematured at 100°C for 5 min, and incubated with 0.3 M NaOH (final concentration) for 20 min. After addition of 10 μl volumes of bisulfite/hydroquinone mix (0.5 M) sodium hydroxymetilate (Adrich, nos. 24-39-53, 0.1 I M hydroquinone (Sigma, no. J 89400)), and 0.4 M NaOH, the solution was overlaid with mineral oil and incubated for 5 h at 55°C. Bisulfite-treated DNA was precipitated with isopropanol (with 50 μg of glycogen), desulfitonied with NaOH (0.3 M final concentration) for 15 min at 27°C, purified, and amplified by PCR. Primers for amplification (underlined) [AAATATT TTATATTGTTT TTT TTT] and shoh [AACCCCTCACTA TTACCAT] were designed with Methprimer (23). PCR-amplified DNA was cloned using a TOPO cloning kit (Invitrogen). Data in the paper were derived from sequencing 14 or 15 clones. **Blue native electrophoresis and histochemical staining.** Blue native electrophoresis was performed as described elsewhere (36). Briefly, to solubilize respiratory complexes, equal protein amounts of purified mitochondrial sample were supplied with 3.5 volumes of buffer C (1 M ammonium acetate, 50 mM BisTris-HCl (pH 7.4)) and 1 volume of 10% deoxycholate. The samples were centrifuged at 100,000 × g for 15 min. The supernatant protein concentration was measured using a Bio-Rad D, kit. The samples were then supplemented with 1/10 volume of 5% Serva Blue G in 1 M ammonium acetate and loaded (90 μg of protein per lane) on a gradient (5 to 13%) native polyacrylamide gel. Catalytic staining was performed as described previously (29). Briefly, to stain for complex I (NADH) activity, the gel was incubated in a solution containing 2 mM MTris-HCl (pH 7.4), 0.1 mg/ml NADH, and 2.5 mg/ml nitroblue tetrazolium on a shaking platform until the gel developed maximal staining. Complex IV activity was evaluated in 25 ml of solution containing 12.5 mg 3,3'-diaminobenzidine tetrahydrochloride, 0.05 M phosphate buffer (pH 7.4), 20 μg/ml catalase, 25 mg cytochrome c, and 1.87 mg/ml ascorbic acid. After staining, the gels were fixed in 50% methanol and 10% acetic acid for 15 min. **Production and analysis of Uqcr1-overexpressing cells.** N2A cells were cultured in Dulbecco modified Eagle medium plus 10% fetal calf serum plus nonessential amino acids (Invitrogen). The mouse Uqcr1 coding sequence was PCR amplified from cDNA (Clontech MARATHON Ready mouse 11-day embryonic cDNA) and cloned into pBabe Puro (EcoRI, SalI) plasmid. The resulting clone was confirmed by sequencing and transfected into the Phoenix Eco retrovirus packaging cell line by using calcium phosphate. Viral supernatant was collected 2 days after transfection and used to infect N2A cells in the presence of 4 μg/ml Polybrene. Puramycin (4 μg/ml) was applied 48 h after infection and maintained during cell culture. The medium was replaced with puramycin-free medium 24 h before the collection was performed. For Western blotting, cells were boiled in SDS loading buffer and proteins were resolved on a 10% SDS-polyacrylamide gel. Uqcr1 and protein markers were detected simultaneously using mouse monoclonal antibodies (anti-Uqcr1, Molecular Probes A21362) and antiprotein (anti-Uqcr1, Molecular Probes A21362). Measurements of mitochondrial respiration in neuroblastoma cells. Mitochondrial oxygen consumption was measured in permeabilized neuroblastoma (N2A) cells essentially by the method of Holmst et al. (12). Cells were harvested by trypsinization diluted 1:5 in medium A (250 mM sucrose, 10 mM MgCl2, 20 mM HEPES [pH 7.1]), and spun down at 250 × g for 2 min. Recovered cells were resuspended in 1 ml medium A plus 1 ml of 200 μg/ml digitonin. Cells were incubated at 37°C for 1 min prior to a 0.10/dilution in medium A and spun down.**
at 250 ± g for 3 min. The final pellet was resuspended in 1 ml medium A and triturated using five passes through a fire-polished glass pipette to separate the cells. Cell counts were performed in triplicate for each sample, using a hemocytometer. Cell viability was tested using trypan blue. Typically >95% of cells stained with trypan blue following digitonin permeabilization. Permeabilized cells (4 × 10⁶) were introduced into the electroplastic chamber to a final volume of 3 ml in N2A respiration buffer (medium A supplemented with 1 mM ADP and 2 mM KH₂PO₄). Following acquisition of a stable baseline, the complex I substrates cyanide and malate were added to concentrations of 5 mM and 25 mM, respectively before complex I was inhibited by addition of rotenone (2 μM). The complex III substrate succinate was then added (15 mM) and respiration measured before maximal inhibition of the chain at complex III by using myxanthol (50 nM). Finally, the complex IV substrate TMPD (80 μM) was added together with ascorbate (10 mM) and a rate determined prior to complete inhibition of the chain at complex IV by addition of KCN (1 mM).

RESULTS

Detection of misregulated genes by differential display. Mecp2-null mice are normal at birth but develop symptoms at around 6 weeks of age, leading to death after approximately 10 weeks (5, 10). As the time of onset of these phenotypic effects shows considerable variability among individuals, we selected mice according to symptom stage rather than chronological age, using the criteria shown in Table 2. In order to maximize our ability to detect alterations in the expression of low-abundance transcripts, we compared mRNA populations in mutant and wt brains by using a variant of differential display called ADDER (17). We detected at broad a range of mRNAs as possible by using multiple primer sets covering all possible nucleotide combinations flanking the 3′ poly(A) and 5′ adaptors of the mRNA. ADDER is reported to detect transcripts present at ~10 copies per cell and to be sensitive to small differences in RNA levels (17). We performed ADDER on three pools (three brains per pool) of total RNA from brains of "late-symptomatic" Mecp2-null mice (Table 2), using as controls three equivalent pools from brains of wt littermates. In total, we used 192 primer combinations, theoretically allowing detection of about 10,000 RNA species. We observed 39 bands that were more intense in Mecp2-null samples than in the wt and 11 bands that were less intense. Bands corresponding to 36 nonregulative genes were recovered and tested for aberrant gene expression by quantitative real-time PCR. Significant deregulation of gene expression in late-symptomatic brains was verified for 12 genes (Table 3). We next asked whether these genes are aberrantly expressed in Mecp2-null mice that had only recently begun to display symptoms ("early-symptomatic" mice [Table 2]). Quantitative PCR on three pools of Mecp2-null brains (three brains per pool) revealed 3 out of the 12 genes to be misexpressed in brains of early-symptomatic mice, in comparison with age-matched controls (t test, P ≤ 0.05). All of the identified genes were expressed apparently normally in presymptomatic Mecp2-null brain RNA (Table 3).

The three genes that were misexpressed in early symptomatic animals were considered more likely to be primary con-

### Table 2. Classification of Mecp2-null mice according to the manifestation of phenotype

<table>
<thead>
<tr>
<th>Symptom stage</th>
<th>Manifestation of phenotype</th>
<th>Age (days)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presymptomatic</td>
<td>-</td>
<td>-80</td>
</tr>
<tr>
<td>Early symptomatic</td>
<td>/</td>
<td>55</td>
</tr>
<tr>
<td>Late symptomatic</td>
<td>+</td>
<td>-70</td>
</tr>
</tbody>
</table>

*Average age of mice displaying the symptoms.

### Table 3. Genes found by a variant of differential display and confirmed by real-time PCR

<table>
<thead>
<tr>
<th>Gene accession no.</th>
<th>Gene (protein or comment)</th>
<th>Presymptomatic</th>
<th>Early symptomatic</th>
<th>Late symptomatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_025407</td>
<td>Lqtrc (ubiquinol-cytochrome c reductase core protein 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK087448</td>
<td>Unknown (contains SAM domain)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC028971</td>
<td>Gld2 (implicated, maternally expressed)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK031516</td>
<td>Hith2b (histone 1 H2b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK034339</td>
<td>Unknown (similar to estrogen/ lipase/koestrerol family members)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AU038081</td>
<td>mt-ND2 (NADH dehydrogenase 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_175092</td>
<td>Rboh (rust homolog gene family, member f)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK09648</td>
<td>Unknown</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK028999</td>
<td>Ccl6 (cell adhesion molecule-related down-regulated by oncogenes)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC051510</td>
<td>Ccl9 (chemokine [C-C motif] ligand 19)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC058513</td>
<td>Smp70 (14 kDa small nuclear ribonucleoprotein/ribonucleoprotein A)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mean values are normalized against the GAPDH gene and expressed relative to wt values. Significance was determined by the Student t test. Boldface indicates genes that are significantly up regulated (P < 0.05).
tributors to the observed pathology than those expressed only in terminally ill animals. One of the three genes is a putative member of the esterase/lipase/thiosterase gene family. This gene is expressed in several tissues and has 43% amino acid sequence identity with arylaacetamide deacylase, which plays a role in lipid metabolism. A second gene was of unknown function, as no annotated relatives could be detected. In this study, we focused on the third early misexpressed gene, which encodes Uqcr1, a core subunit of complex III which is part of the mitochondrial electron transport chain. Interestingly, the gene for NADH dehydrogenase subunit 2 (nrd2), another component of the mitochondrial electron transport chain (complex I), was found among the 11 genes that were misregulated in late-symptomatic animals. Uqcr1, a nucleic-encoded gene, was significantly up-regulated in early- and late-symptomatic brains, whereas mitochondrially encoded mt-Nd2 was significantly down-regulated in late-symptomatic, but not early-symptomatic, brains (Fig. 1a and b; Table 3). We have failed to detect any difference between the amounts of Uqcr1 protein present in the brains of MeCP2-null (early- and late-symptomatic) mice and wt littermates by Western blotting (data not shown; see Discussion).

There are several possible reasons why the set of genes detected by differential display did not overlap with those reported in previous studies. Theoretically ADDER permits expression analysis of about 80% of polyadenylated transcripts, but in practice this number is considerably lower. Our best estimate was that we examined expression of up to 10,000 transcription units, which is around half of the total number of expressed sequences in mouse brain and may therefore exclude some reported targets. An additional limitation of the technique is that the PCR amplification step may be unrepresentative, leaving some genes undetected. The microarray experiment performed by Nuber et al. (31) utilized an array of 13,000 genes (about 60% of the expected total), and changes of less than twofold were disregarded. As most of the genes found by ADDER differential display are misregulated less than twofold, they may be missed by the microarray data analysis. Re-examining the microarray data set, we found that Gtfl/Meg3 shows up-regulation of 1.6-fold. This gene therefore is found in both analyses.

**MeCP2 binds to the Uqcr1 promoter in vivo.** The delayed onset of Uqcr1 up-regulation may mean that misexpression is an indirect consequence of the absence of MeCP2. However, the amount of MeCP2 in neurons is known to increase dramatically as neurons mature (15, 26), and therefore some genes that are not initially affected by MeCP2 may come under its regulatory influence as MeCP2 becomes more abundant. The increase in the concentration of MeCP2 in murine brain occurs progressively during postnatal life as synaptogenesis proceeds and neurons mature (16, 26). If Uqcr1 is a direct target, MeCP2 should bind in the vicinity of its promoter. This prediction was verified by chromatin immunoprecipitation, which showed that an anti-MeCP2 antibody could precipitate a DNA region near the Uqcr1 transcription start site (Fig. 2).

This region was not detected when MeCP2-null brain nuclei were subjected to immunoprecipitation, thereby eliminating the possibility of nonspecific cross-reaction of the antibody with other nuclear components (Fig. 2b). A known MeCP2 binding site, promoter III of the Bdnf gene (6, 24), served as a positive control for the immunoprecipitation reaction. To control for the equivalence of MeCP2-null brain chromatin preparations, we found that an antibody against dimethylated lysine 9 of histone H3 gave identical recovery of the Uqcr1 promoter region in MeCP2-null and wt brain nuclei. We also tested for the presence of methyl-CpG sites in the region that appeared to bind MeCP2 by using bisulfite sequencing. One of a group of CpGs flanking the Uqcr1 promoter CpG island was found to be 33% methylated in total brain DNA (Fig. 2a). We concluded that MeCP2 is associated with the Uqcr1 gene in brain and may directly influence its expression, although we cannot exclude the possibility that Uqcr1 overexpression is an indirect consequence of MeCP2 deficiency.

**Mitochondrial abnormalities in the MeCP2-null mouse brain.** We speculated that the abnormal expression of Uqcr1 in symptomatic MeCP2-null mice might affect mitochondrial morphology and/or physiology. Initial examination of purified mitochondria by electron microscopy did not reveal gross structural differences between wt and MeCP2-null mice (Fig. 3a). We therefore performed a polarographic oxygen electrode study to evaluate the activities of different complexes within the electron transport chain (Fig. 3b). The protonophore FCCP uncouples the activity of complexes I to IV from the rate-limiting electrochemical proton gradient, thereby permitting maximal activity. Symptomatic MeCP2-null mitochondrial samples consistently showed increased uncoupled respiration rates when substrates were fed into the respiratory chain upstream of complex III (Fig. 3c, PM and Succ.)
but not with a substrate entering downstream of complex III (Fig. 3c, TMPD). This difference was not seen in brain mitochondria from presymptomatic mice (Fig. 3c, left panel). The data suggest that in symptomatic Mecp2-null mice the maximal capacity of the respiratory chain upstream of complex IV is increased. This is confirmed by the observation that differences in state 3 respiration rates between symptomatic Mecp2-null and wt mice were also observed only for substrates that feed into the respiratory chain upstream of complex IV (Fig. 3f).

Under physiological conditions, the proton translocation associated with complexes I, III, and IV works against an electrochemical proton gradient, providing a mechanism for respiratory control. The coupled states 2 and 4 represent this condition. Increases in state 2 and 4 oxygen consumption were observed between symptomatic Mecp2-null and wt mitochondria. This increase was not observed in presymptomatic Mecp2-null mice.
Mitochondria when substrates were fed in at complexes I and II but not when these were bypassed by addition of a complex IV substrate (Fig. 3d and e). Again, these differences were not seen in presymptomatic mice.

Measurement of the amount of oxygen consumed during the conversion of a known amount of ADP (during state 3) allows the calculation of the ATP/O ratio. This relates the stoichiometries of protons translocated per electron flowing down the respiratory chain to the number of protons flowing through the ATP synthase per ATP molecule produced. No significant differences were observed in any of the measured ATP/O ratios (Fig. 3g), implying that the respiration rate differences reported above were not the result of a change in the respiratory chain proton pumps or the efficiency of ATP synthase.

Our results show that respiration rates for symptomatic Mecp2-null animals are significantly increased relative to these for wt controls for all substrates that feed in upstream of complex IV. Examination of the respiratory control ratios indicates that the rates of coupled respiration have increased proportionally more than the uncoupled rates (Fig. 3h). This probably indicates an increase in the non-OHmic proton conductance across the mitochondrial inner membrane (30). To maintain the proton gradient against this background “leak,” the electron transport chain works faster and therefore consumes more oxygen. Thus, mitochondria from symptomatic mutants appear to have an overall greater respiratory capacity (Fig. 3c) but also appear to work less efficiently (Fig. 3e and h).

Complexes I and II transfer electrons independently to complex III via a common pool of ubiquinone. Despite the independent nature of the electron paths, the observed genotype-specific respiratory effects were similar with substrates specific for either complex. The common link between the two pathways is complex III. We could not rule out the possibility, however, that the observations are due to elevated activities of both complexes I and II. We tested this possibility for complex I by using blue native electrophoresis, which allows activity-based visualization of each respiratory complex (36). Coomassie blue staining of respiratory complexes from wt and Mecp2-null mouse brains showed apparently normal levels (Fig. 4a and data not shown). We next assayed the enzymatic activities of complexes I and IV in the gel by using histochemical staining for NADH dehydrogenase and cytochrome oxidase (39). Complexes I and IV each produced a single band of the expected size (Fig. 4b and c). Samples derived from three wild-type and mutant littermate pairs were quantified using densitometry. The results showed that complex I activity was indistinguishable between wt and mutant brain mitochondria (t test, \( P > 0.05 \)). This result argues against the possibility that complex I is responsible for the increase in respiration rates and therefore further implicates complex III. As there is currently no in-gel assay for complex III activity, this hypothesis was not directly testable. An interesting by-product of the blue native electrophoresis analysis was the discovery of a reproducible decrease in the activity of complex IV (t test, \( P < 0.001 \)) in samples derived from Mecp2-null mouse brains (Fig. 4d).

Uqcr1 overexpression causes abnormal mitochondrial respiration in N2A cells. Multiple events could potentially cause the mitochondrial respiration abnormalities in Mecp2-null mouse brain described above. The simplest hypothesis, however,
ever, is that overexpression of Uqcrcl alone is responsible for the measured increase in oxygen consumption with complex I and II substrates. To test this hypothesis, we overexpressed Uqcrcl in neuroblastoma cells. The Uqcrcl gene was introduced into mouse N2A cells by using retroviral infection, and infected cells were maintained and expanded by selecting for the vector-carried puromycin resistance gene. Stable Uqcrcl-overexpressing cell lines produced 2.8 times more RNA and 1.6 times more Uqcrcl protein than the control cells infected with vector only (Fig. 5a and b). Permeabilized cell suspensions of Uqcrcl-overexpressing N2A cells and control cells were then used for respiration analysis with a Clark oxygen electrode as described above. When substrates upstream of complex III were used (Fig. 5c, pyruvate/malate and succinate), we observed a significant increase in the uncoupled respiration rate in the Uqcrcl-overexpressing cell line (t test, $P_{\text{pyruvate/malate}} < 0.001$ and $P_{\text{sucinate}} < 0.05; n = 19$). Respiration rates with a complex IV substrate (Fig. 5c, TMPD/ascorbate) resulted in similar rates of oxygen consumption in both cell lines (t test, $P > 0.05$). Thus, artificial overexpression of Uqcrcl strikingly reproduces the data obtained with isolated uncoupled mitochondria from the brains of Mecp2-null mice discussed above (compare Fig. 3 and 5).

**DISCUSSION**

**Relationship of Uqcrcl overexpression to mitochondrial abnormalities.** The data presented here show that MeCP2 binds to the promoter of the Uqcrcl gene in vivo and that Uqcrcl mRNA expression is elevated in brains of Mecp2-null mice that have acquired neurological symptoms. Uqcrcl up-regulation correlates positively with symptom severity and with a significant increase in mitochondrial respiratory capacity and a reduction in respiratory efficiency. The defect appears to be associated with respiratory complex III, which contains the Uqcrcl protein, as increased respiration was seen when appropriate substrates were provided to either complex I or complex II. Blue native electrophoresis showed normal levels of complex I activity in the mutant, indicating that this complex is not responsible for the observed increase in respiration. As these findings implicated complex III as the source of enhanced respiratory activity, we asked whether overexpression of Uqcrcl alone is sufficient to increase mitochondrial respiration. Indeed, a cell line overexpressing Uqcrcl showed increased respiration rates with substrates upstream of complex IV. This argues that the mitochondrial respiratory abnormalities seen in Mecp2-null brain are a consequence of Uqcrcl overexpression.

Although the evidence strongly supports a causal relationship between Uqcrcl overexpression and abnormalities in mitochondrial respiration, we have not been able to detect reliable increases in brain Uqcrcl protein due to the ~1.7-fold increase in Uqcrcl mRNA in the symptomatic Mecp2-null brain. Two possible explanations for this discrepancy can be considered: (i) Uqcrcl may be subject to tight posttranscriptional regulation, for example, by feedback modulation of mRNA translation efficiency or by degradation of excess unassembled protein, or (ii) Western blotting may be insensitive to quantitative changes of such low magnitude. The finding that a 2.8-fold up-regulation of Uqcrcl mRNA in N2A cells led to only a 1.6-fold up-regulation of protein is relevant. A comparable ratio of excess mRNA to excess protein in the brain samples would lead to a 1.2-fold increase in Uqcrcl protein, which would be difficult to detect by Western blotting.

While the molecular origin of these respiratory abnormalities remains unclear, we speculate that the exquisite sensitivity of mitochondrial assembly to the stoichiometry of its constituent proteins is responsible. For example, increased amounts of Uqcrcl may destabilize complex III assembly, leading to faster electron transfer or perhaps transfer by Uqcrcl alone. Another possibility is that Uqcrcl interacts with a complex III inhibitor, which would be titrated by excess Uqcrcl. In the structure of complex III (cytochrome $b_6$), Uqcrcl is oriented towards the matrix, which might allow interactions of this kind (14).

Our studies uncovered other mitochondrial defects in the Mecp2-null mouse brain: (i) significantly elevated coupled res-
piration rates, suggesting increased proton conductance across the inner mitochondrial membrane; (ii) reduced activity of cytochrome oxidase, implying a defect in complex IV; and (iii) down-regulation of mRNA for NADH dehydrogenase, a mitochondrially encoded component of complex I, in late symptomatic animals. The observed increase in coupled respiration of up to 30% indicates a loss of respiratory control which may have a significant impact on mitochondrial efficiency in the Mecp2-null mice. The other changes are subtle (<2-fold) but may reflect suboptimal mitochondrial performance in brains of symptomatic Mecp2-null mice. Whether these effects are related to Uqcrcl overexpression or arise independently is not currently known.

Mitochondrial abnormalities and RTT. The notion that mitochondrial abnormalities play a role in RTT predates the discovery of the genetic origin of the condition. With the realization that McCP2, a nuclear transcriptional repressor, is mutated in RTT, the mitochondrial link became less compelling. It was recently noted, however, that a patient with symptoms normally associated with mitochondrial disorders (hypotonia, small stature, developmental delay, and a slight decrease in respiratory chain enzyme activity) harbored mutations in the MEC2P gene (11). This overlap between symptoms of RTT and mitochondrial disorders recalls early reports of structural abnormalities (4, 8, 9, 34) and defects in the electron transport chain (7, 8) in mitochondria from skin and muscle biopsies of RTT patients. Moreover, about half of RTT patients were reported to have elevated levels of circulating lactic or pyruvic acid, which might be caused by defects in the efficiency of the respiratory chain and ura cycle complexes, both of which are mitochondrial (21, 25).

In humans many disorders affecting the brain are due to mutations in nuclear or mitochondrially encoded components of the mitochondrial, sometimes resulting in increased oxidative stress or induction of neuronal apoptosis. As Rett syndrome is not a neurodegenerative disorder (3), any contribution of mitochondrial dysfunction to RTT symptoms may take the form of chronic mitochondrial underperformance, rather than catastrophic failure leading to neuronal death. The changes that we have observed in the mouse model of RTT appear to meet this criterion, as they are small in magnitude and may therefore compromise brain function without precipitating cellular death. Future work will probe the involvement of McCP2 in the expression of proteins that are targeted to the mitochondrion and will test the hypothesis that aberrant mitochondrial function as seen in mice has a role to play in RTT.

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REFERENCES


