Investigating the role of
Amyloid Precursor – Like Protein 2
in Motor Neurone Disease

Phan Hong Truong

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Department of Pharmacology & Therapeutics

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Abstract

Motor neurone disease (MND) is a fatal human neurodegenerative disorder. The most common form of MND is amyotrophic lateral sclerosis (ALS). MND is characterised by the progressive destruction of motor neurons in the central nervous system which causes muscle weakness, muscle atrophy, paralysis and ultimately death. The sporadic forms of the disease account for the majority of patients, and 5-10% of MND cases are inherited (familial MND) (Marin et al., 2017). Both sporadic and familial MND share similar clinical and pathological features, suggesting common molecular mechanisms of degeneration. Among the familial MND patients approximately 20% possess a mutation in the SOD1 gene encoding for the enzyme Cu/Zn superoxide dismutase (Rosen et al., 1993). There are more than 170 different SOD1 gene mutations described, and the majority are missense substitutions resulting in a toxic gain of enzyme function (http://alsod.iop.kcl.ac.uk/). Transgenic mouse models over-expressing mutant forms of the human SOD1 gene replicate key pathological symptoms seen in MND patients and are widely used to study MND. Despite progress in deciphering the molecular mechanisms of this disease, the cause and modulation of MND remains unclear.

The Amyloid Precursor Protein (APP), is well-known for its association with Alzheimer's Disease, and it has been shown to be a modulator of MND. APP protein expression levels were increased in the spinal cords from MND patients as well as in SOD1 transgenic mice at symptomatic stage of the disease (Koistinen et al., 2006; Rabinovich-Toidman et al., 2015). The resultant SOD1-G93A:APP/- mice from the cross breeding between APP homozygous deletion and SOD1-G93A transgenic mice (overexpress human SOD1 gene with G93A familial mutation) showed significant decrease in MND pathogenesis and reduced disease progression (Bryson et al., 2012). The SOD1-G93A:APP/- mice also displayed significantly ameliorated muscle contractility, improved neuromuscular junction innervation and decreased motor neuron loss. Taken together these findings suggest an important role for APP in MND pathophysiology.

APP is part of a gene family that includes the amyloid precursor-like protein 1 (APLP1) and amyloid precursor-like protein 2 (APLP2) genes. To understand if other APP-family members modulated MND we investigated the role of APLP2 in the SOD1-G37R
transgenic mouse model. We found a significant sex-dependent increase in the expression of APLP2 protein in the spinal cord of the SOD1-G37R mice. To test if APLP2 gene expression can modulate disease outcomes in MND we crossed the SOD1-G37R and APLP2 knockout (KO) mice to generate the SOD1:APLP2+/- and SOD1:APLP2-/- lines. We found the lack of APLP2 expression improved motor performance and extend survival in a sex-dependent manner. The molecular basis for APLP2’s actions identified effects on muscle physiology and synaptic function at the neuromuscular junction.

Taken together, our novel results demonstrate there are sex-dependent differences in the SOD1 mouse model, and this is affected by APLP2 expression. These data extend the modulatory role by the amyloid precursor protein family in MND, and identify the APP-family as an important target for further investigation into the cause and regulation of MND.
Declaration

I declare that:

1. The thesis comprises of my original work towards the PhD except where indicated in the Preface

2. Due acknowledgement has been made in the text to all other material used

3. The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices

Phan Hong Truong
Department of Pharmacology & Therapeutics
The University of Melbourne
Parkville, VIC, 3010
Australia
Preface
I acknowledge that specific experiments outlined below were included in this thesis and performed by others:

- The identification of sex hormones in the human and mouse APLP2 gene was carried out in conjunction with fellow PhD student Chaitanya Inampudi (Chapter 5, Section 5.2.13)
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Publications & Conferences

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Conference and meetings- poster presentations

1. Student Brain Symposium (Students of Brain Research), 2014, Melbourne, Victoria, Australia.
2. International Society of Neurochemistry meeting, 2015, Cairns, Queensland, Australia.
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### Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AChRs</td>
<td>Acetylcholine receptors</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloprotease</td>
</tr>
<tr>
<td>AICD</td>
<td>APP intracellular domain</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ALSTDI</td>
<td>ALS Therapy Development Institute</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APLP-1</td>
<td>Amyloid precursor-like protein 1</td>
</tr>
<tr>
<td>APLP-2</td>
<td>Amyloid precursor-like protein 2</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ARE</td>
<td>Androgen responsive element</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenylpyrophosphatase</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>BACE1</td>
<td>Beta site APP cleaving enzyme 1</td>
</tr>
<tr>
<td>BACE2</td>
<td>Beta site APP cleaving enzyme 2</td>
</tr>
<tr>
<td>Bak</td>
<td>BCL2 antagonist/killer</td>
</tr>
<tr>
<td>Bax</td>
<td>BCL2-associated X protein</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BTX</td>
<td>Bungarotoxin</td>
</tr>
<tr>
<td>C83</td>
<td>83 amino acid residues APP fragment</td>
</tr>
<tr>
<td>C99</td>
<td>99 amino acid residues APP fragments</td>
</tr>
<tr>
<td>C9ORF72</td>
<td>Chromosome 9 open reading frame 72</td>
</tr>
<tr>
<td>CHAT</td>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSA</td>
<td>Cross sectional area</td>
</tr>
<tr>
<td>CTCI</td>
<td>Corrected total cell intensity</td>
</tr>
<tr>
<td>CTF</td>
<td>C terminal fragments</td>
</tr>
<tr>
<td>CuBD</td>
<td>Copper binding domain</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor digitorum longus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
</tbody>
</table>
ER  Estrogen receptor
ERE  Estrogen response element
FF  Fast-twitch fatigable
FFR  Fast-twitch fatigue resistant
FITC  Fluorescein isothiocyanate
FTD  Frontotemporal dementias
FTLD  Frontotemporal lobar degeneration
FUS  Fused in sarcoma
GA  Gastrocnemius
GFAP  Glial fibrillary acidic protein
GFLD  Growth factor like domain
GFP  Green fluorescent protein
GWAS  Genome-wide association study
FTD  Frontotemporal dementias
FTLD  Frontotemporal lobar degeneration
FUS  Fused in sarcoma
GA  Gastrocnemius
GFAP  Glial fibrillary acidic protein
GFLD  Growth factor like domain
GFP  Green fluorescent protein
GWAS  Genome-wide association study
HBD  Heparin binding domain
HCl  Hydrochloric acid
HRP  Horseradish peroxidase
iPSCs  Induced pluripotent stem cells
KPI  Kunitz protease inhibitor
MHC  Myosin heavy chain
MHC-I  Myosin heavy chain class I
MND  Motor neurone disease
MRI  Magnetic resonance imaging
mRNA  Messenger RNA
NMJ  Neuromuscular junction
NRG-1  Neuregulin 1
OCT  Optimal Cutting Temperature
OPTN  Optineurin
PBS  Phosphate buffered saline
PBST  Phosphate buffered saline tween
PCR  Polymerase chain reaction
PDL  Poly-D-lysine
PFA  Paraformaldehyde

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PGC-1α  Peroxisome proliferator-activated receptor-γ coactivator-1α
PLA        Plantaris
PNS        Peripheral nervous system
PR         Progesterone receptor
PRE        Progesterone responsive element
RNA        Ribonucleic acid
ROI        Regions of interest
RT         Room temperature
sAPPα      Soluble α-cleaved APP fragment
sAPPβ      Soluble β-cleaved APP fragment
SDS        Sodium dodecyl sulfate
SDS-PAGE   Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SECS       Seconds
SEM        Standard error of the mean
SFR        Slow twitch fatigue resistant
SOD1       Superoxide dismutase 1
SOL        Soleus
TA         Tibialis anterior
TAE        Tris/Acetate/EDTA
TARDBP     Transactive response DNA binding protein 43 kDa
TBI        Traumatic brain injury
TBS        Tris buffered saline
TBST       Tris buffered saline tween
TCEP       Tris-(2-carboxyethyl) phosphine
TDP-43      Transactive response DNA binding protein 43 kDa
TIF        Tagged Image File
TRIS       Trisaminomethane
UBQLN2     Ubiquilin-2
UPS        Ubiquitin proteasome system
WT         Wild type
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Chapter 1: Introduction

1.1 Motor Neurone Disease

Motor neurone disease (MND) refers to a class of progressive neurological disorders characterised by the degenerative processes of the upper and/or lower motor system. The term encompasses several conditions including Amyotrophic Lateral Sclerosis (ALS), spinal bulbar muscular atrophy, progressive bulbar palsy, progressive muscular atrophy and primary lateral sclerosis. The most prevalent form of MND is ALS, therefore in this thesis, the term MND is used synonymously for ALS.

MND was first described by Jean-Martin Charcot, a French neurologist, in 1868 in a group of patients suffered from progressive muscular atrophy (Charcot 1874). Charcot described the patient as exhibiting signs of weakness and atrophy (symptoms of lower motor neurons deficit) with spasticity and contracture (symptoms of upper motor neurons deficit) (Goetz, 2000). The term “amyotrophic” refers to atrophy of the muscle fibres and muscle weakness, while “lateral” relates to the nerve pathways of both sides of the spinal cord and “sclerosis” is “hardening” of the spinal cord corresponding to the degeneration corticospinal tract.

Classical MND is now being described as a progressive and fatal neurodegenerative disorder that results in the selective destruction of the upper (brain cortex) and lower motor neurons (brain stem and ventral horn of the spinal cord) (van Es et al., 2017). This inevitably leads to muscle weakness, paralysis and eventually death. The majority of MND cases are found in the age group between 40-70 years. The average life expectancy of an MND patient is between 2-5 years following clinical diagnosis (International alliance ALS/MND associations, 2015). The incurable and fatal outcome of the disease urges an increase in research efforts to understand the cause(s) of this disease, in the hope to slow down disease progression and ultimately find a cure.

1.1.1 Epidemiology, diagnosis and treatment of MND

The worldwide prevalence of MND is 3-5 individuals per 100,00 people and approximately 140,000 people are currently diagnosed with MND annually (International alliance ALS/MND associations, 2015). In Australia, more than 2,000 people are currently diagnosed with MND and the incidence is higher among men (60%) than women (40%) (MND Australia association,
The disease is often presented in adulthood around 55-70 years of age, however a small percentage of the cases (~5%) are seen in individuals before the age of 30, and in extremely rare cases patients are diagnosed before the age of 20 (Aggarwal and Shashiraj, 2006; Li et al., 1988).

The predominant symptoms of MND include muscle weakness caused by the destruction of the motor neurons in the ventral horn of the spinal cord and the brain stem that extends the transmission of axons synapsing with the muscle. In certain cases of MND, depending on the motor nuclei involved, can result in symptoms such as difficulty moving the limbs and sustaining posture, speaking and breathing. In terms of the site of disease onset, a small proportion of MND patients are described to have ‘bulbar onset’ (25-30%), a condition where the patient shows initial symptoms of difficulty speaking clearly, and speech becomes garbled and slurred (Al-Chalabi et al., 2016; Shellikeri et al., 2017). Thereafter the patient will have difficulty in swallowing and lose tongue mobility and subsequently has complete loss of speech (Haverkamp et al., 1995). While the remaining ~70% of patients are described to have ‘spinal onset’ with symptoms initially affecting one of the legs and patients experience weakness during walking or running and they tend to trip or stumble, while there are patients who show symptoms first in their hand or arm and they often experience problems with daily tasks such as writing, buttoning their shirt or turning a door handle (Gordon, 2013; Haverkamp et al., 1995; Shellikeri et al., 2017).

All types of MND culminates in the degeneration of both upper and lower motor neurons, with respiratory failure or pulmonary complications being the principal cause of death. Ideally, diagnosing a patient with MND in its pre-symptomatic clinical stage will be most advantageous as this will provide opportunities for early intervention therapies that could prevent significant neuronal loss and associated complications. But as of now, there is no consensus on the best biomarker test to be used for diagnosing MND. Moreover, to date, there is no cure for MND and treatment options are symptomatic and focused on palliative care. Currently, the only treatment of MND includes Riluzole and Edaravone, both are the only Food and Drug Administration approved drugs and help to alleviate the symptoms of MND (Bensimon et al., 1994; Lacomblez et al., 1996; Miller et al., 2009a, b). The first approved drug is Riluzole, which only prolongs patient life by 2-3 months. The exact mechanism of action by Riluzole is
unclear, but it has been shown that the drug is more effective among younger patients or patients at an early stage of diagnosis (Bensimon et al., 1994; Lacomblez et al., 1996).

### 1.1.2 Sex differences in MND

The incidence of MND is higher among men than women with a ratio of 1.5:1 male to female patients (McCombe and Henderson, 2010; Zoccolella et al., 2008), with a higher prevalence in younger male patients and this difference, increases in the older age groups (Manjaly et al., 2010). While no clear effects on survival were identified, sex differences were observed in the clinical features of the disease. Men were found to have a greater likelihood of onset in the spinal regions, while bulbar onset was greater in women (Millecamps et al., 2010). When treated as an independent predictor of MND, sex did not have an overall effect (del Aguila et al., 2003; Mandrioli et al., 2006; Talman et al., 2009; Zoccolella et al., 2008). Recent neuroimaging studies in MND cases indicated significant sex-dependent differences in the anatomical patterns of cortical and subcortical regions (Bede et al., 2014). The exact factors contributing to the sex differences in MND remains to be elucidated. However, reports have indicated a higher risk of MND in females with later menarche and earlier menopause (Chio et al., 1991; Chio et al., 2009c), while a lower risk of MND occurred in females with a history of hysterectomy (Popat et al., 2006). These data suggest oestrogens may be protective against MND (de Jong et al., 2013).

In addition, sex differences were observed to modify the rate of disease progression in transgenic mouse models expressing a familial human SOD1 mutation (Suzuki et al., 2007; Veldink et al., 2003). Earlier-onset of the disease phenotype was observed in males compared to female SOD1-G93A mice (Heiman-Patterson et al., 2005). Estrogen was responsible for the sex differences in the SOD1-G93A mice, as estrogen therapy could reverse the phenotype when the males undergo castration (Choi et al., 2008). Other studies reported that environmental enrichment and increasing physical activity could accelerate the disease progression in females but not in male SOD1-G93A transgenic mice (Stam et al., 2008). These studies support the notion that sex differences can be a modifier for the course of the disease.

### 1.1.3 Risks factors of MND

While a number of risk factors for MND have been proposed to be associated with the development of MND, the only established risk factors are age, sex and family history (Chio et al., 2009b). As such, no environmental risk factor has been proven as a causative factor of
MND. Studies for exogenous risk factors of MND is challenging due to the insufficient power caused by the lack of replication studies and the small sample sizes. A person may be genetically susceptible to the disease if they encounter an exogenous factor, for example, the neurotoxin amino acid β-methylamino-L alanine (BMAA), found in the seeds of *Cycas micronesica* is thought to be responsible for the high incidence of MND in the Guam population (Wang et al., 2011). Other risks factor for the development of MND includes: intensive exercise (Beghi et al., 2011; Chio et al., 2009a; Turner et al., 2012), low premorbid body mass index (BMI) (O’Reilly et al., 2013), smoking (Armon, 2009), traumatic brain injury (Chen et al., 2007), environmental exposures to toxins (Fang et al., 2009), military service (Beard et al., 2016) and electric fields exposure (Huss et al., 2015; Vergara et al., 2013; Vergara et al., 2015).

### 1.1.4 Genetics of MND

#### 1.1.4.1 Sporadic forms of MND

Based on the pattern of inheritance, there are two types of MND, familial MND and sporadic MND. The majority of MND cases are classified as sporadic, which occur in the absence of clear genetic linkage. Sporadic MND is believed to arise from the complex interactions between genetic susceptibility and environmental factors. The incidence of sporadic MND in Western countries range between 1-3 per 100,000 (Chio et al., 2013) and the lifetime risk of MND is 1 in 400 individuals (Johnston et al., 2006). Clinical presentations of familial and sporadic MND cannot be distinguished, apart from the mean age of onset, which for sporadic is ~57 years, while 10 years earlier for familial cases (Consortium, 2013; Roosli et al., 2007).

#### 1.1.4.2 Familial forms of MND

Approximately 5-10% of MND cases are classified as familial. Often, MND cases are inherited in an autosomal dominant manner, and only rarely does it exist as a recessive trait or X-linked (Gros-Louis et al., 2006; Ticozzi et al., 2011). Among the familial MND cases, approximately 30-40% is caused by hexanucleotide repeat expansion mutation in the intron of C9ORF72 gene (Hodges, 2012) and about 20% (Andersen, 2006) is caused by mutations in the Cu/Zn superoxide dismutase 1 (SOD1) gene (Figure 1.1 Major responsible genes for sporadic and familial cases of MND.). To date, remarkably over 170 different disease associated mutations have been identified in the SOD1 gene (http://alsod.iop.kcl.ac.uk/). A description of each MND associated gene is beyond the scope of this thesis and I will focus on the more well described
genes. Table 1.1 summarises the genes associated with familial cases of MND discovered to date.

**Figure 1.1 Major responsible genes for sporadic and familial cases of MND.**

MND is divided into 2 forms sporadic (accounts for 90-95%) and familial (5-10%). The two most common genetic causes of MND are Chromosome 9 Open Reading Frame 72 (C9ORF72) and Superoxide Dismutase 1 (SOD1). C9ORF72 accounts for ~30-40% of patients with familial MND while SOD1 mutations account for ~20% of patients. TARDBP and FUS account for ~4% of familial cases, while others were identified in <1% of familial cases. Figure adapted from (Turner et al., 2013).
Table 1.1 Genes associated with familial cases of motor neurone disease.

<table>
<thead>
<tr>
<th>Function/process</th>
<th>Locus</th>
<th>Gene</th>
<th>Gene name</th>
<th>Chromosome</th>
<th>Onset</th>
<th>Inheritance</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide metabolism</td>
<td>SOD1</td>
<td>SOD1</td>
<td>Cu/Zn superoxide</td>
<td>21q22.11</td>
<td>Adult</td>
<td>Ad</td>
<td>~20</td>
</tr>
<tr>
<td>Endosomal trafficking and cell signalling</td>
<td>ALS 2</td>
<td>ALS2</td>
<td>Alsin</td>
<td>2q33.2</td>
<td>Juvenile</td>
<td>Ar</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Unknown</td>
<td>ALS 3</td>
<td>ALS3</td>
<td>Unknown</td>
<td>18q21</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RNA processing</td>
<td>ALS 4</td>
<td>SETX</td>
<td>Senataxin</td>
<td>9q34.13</td>
<td>Juvenile</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>DNA damage</td>
<td>ALS 5</td>
<td>SPG11</td>
<td>spastic paraplegia 11 (autosomal recessive)</td>
<td>15q14</td>
<td>Juvenile</td>
<td>Ar</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RNA processing (malignant liposarcoma)</td>
<td>ALS 6</td>
<td>FUS</td>
<td>fusion (involved in malignant liposarcoma)</td>
<td>16p11.2</td>
<td>Adult</td>
<td>Ad</td>
<td>4</td>
</tr>
<tr>
<td>Unknown</td>
<td>ALS 7</td>
<td>ALS7</td>
<td>Unknown</td>
<td>20p13</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Endosomal trafficking and cell signalling</td>
<td>ALS 8</td>
<td>VAPB</td>
<td>Vesicle-associated membrane protein-associated protein B</td>
<td>20q13.33</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Neurovasculature</td>
<td>ALS 9</td>
<td>ANG</td>
<td>Angiogenin</td>
<td>14q11.1</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RNA processing</td>
<td>ALS 10</td>
<td>TARDBP</td>
<td>TAR DNA binding protein</td>
<td>1p36.22</td>
<td>Adult</td>
<td>Ad</td>
<td>1-4</td>
</tr>
<tr>
<td>Endosomal trafficking/cell signalling</td>
<td>ALS 11</td>
<td>FIG4</td>
<td>FIG4 homolog, SAC1 lipid phosphatase domain containing (S. cerevisiae)</td>
<td>6q21</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Endosomal trafficking/cell signalling</td>
<td>ALS 12</td>
<td>OPTN</td>
<td>optineurin</td>
<td>10p13</td>
<td>Adult</td>
<td>Ad, Ar</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RNA processing</td>
<td>ALS 13</td>
<td>ATXN2</td>
<td>ataxin 2</td>
<td>12q23-q24.1</td>
<td>Undefined</td>
<td>Undefined</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Ubiquitin/protein degradation</td>
<td>ALS 14</td>
<td>VCP</td>
<td>valosin-containing protein</td>
<td>9p13</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Regulate the degradation of ubiquinated proteins</td>
<td>ALS 15</td>
<td>UBQLN2</td>
<td>ubiquilin 2</td>
<td>Xp11.21</td>
<td>Adult</td>
<td>X-linked</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Endosomal trafficking &amp; cell signalling</td>
<td>ALS 16</td>
<td>SIGMAR1</td>
<td>sigma non-opioid intracellular receptor 1</td>
<td>9p13</td>
<td>Juvenile</td>
<td>Ar</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Endosomal trafficking and cell signalling</td>
<td>ALS 17</td>
<td>CHMP2B</td>
<td>chromatin modifying protein 2B</td>
<td>3p12.1</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cytoskeletal network</td>
<td>ALS 18</td>
<td>PFN1</td>
<td>profilin 1</td>
<td>17p13.3</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>---------------------</td>
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</tr>
<tr>
<td>Cell signalling</td>
<td>ALS 19</td>
<td>ERBB4</td>
<td>v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4</td>
<td>2q33.3-q34</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RNA processing</td>
<td>ALS 20</td>
<td>HNRNPA1</td>
<td>heterogeneous nuclear ribonucleoprotein A1</td>
<td>12q13.1</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Protein binding</td>
<td>ALS 21</td>
<td>MATR3</td>
<td>matrin 3</td>
<td>5q31.2</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Protein degradation, cytoskeletal</td>
<td>ALS-FTD1</td>
<td>C9ORF72</td>
<td>chromosome 9 open reading frame 72</td>
<td>9p21.2</td>
<td>Adult</td>
<td>Ad</td>
<td>~30</td>
</tr>
<tr>
<td>Oxidative phosphorylation and maintenance of Cristae morphology</td>
<td>ALS-FTD2</td>
<td>CHCHD10</td>
<td>coiled-coil-helix-coiled-coil-helix domain containing 10</td>
<td>22q11.23</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>A presynaptic protein essential in synaptic vesicle priming</td>
<td>ALS</td>
<td>UNC13A</td>
<td>unc-13 homolog A (C. elegans)</td>
<td>19p13.12</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>An enzyme with unknown biological function, may act as detoxifying agent</td>
<td>ALS</td>
<td>DAO</td>
<td>D-amino-acid oxidase</td>
<td>12q24</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Endosomal trafficking, axonal transport of vesicles and organelles</td>
<td>ALS</td>
<td>DCTN1</td>
<td>Dynactin</td>
<td>2p13</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cytoskeletal components of myelinated axons, intracellular transport to axons and dendrites</td>
<td>ALS</td>
<td>NEFH</td>
<td>neurofilament, heavy polypeptide 200kDa, heavy chain</td>
<td>22q12.1-q13.1</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cytoskeletal protein</td>
<td>ALS</td>
<td>PRPH</td>
<td>peripherin</td>
<td>12q12</td>
<td>Adult</td>
<td>Ad</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Ad: Autosomal dominant, Ar: Autosomal recessive, DNA: Deoxyribonucleic acid, RNA: Ribonucleic acid

Data obtained from Amyotrophic Lateral Sclerosis Online Database [http://alsod.iop.kcl.ac.uk/](http://alsod.iop.kcl.ac.uk/)
1.1.4.3 Chromosome 9p Open Reading Frame 72 (C9ORF72)

Genome-wide association studies (GWAS) were undertaken to identify MND risk loci. The most robust result from these studies was the locus at Chromosome 9p Open Reading Frame 72 (C9ORF72) (van Es et al., 2009). C9ORF72 is the most common variant associated with familial MND found in ~40% of the cases and ~6 to 8% of sporadic cases of MND (Majounie et al., 2012). Mutations in the C9ORF72 gene involved an expansion of a hexanucleotide repeat sequence GGGGCC in the non-coding region (DeJesus-Hernandez et al., 2011). Typically, normal individuals only carry 2-10 hexanucleotide repeat sequence while affected individuals carry several hundred repeats in the C9ORF72 gene. Mutation in the C9ORF72 gene is the most frequent cause of both FTD and MND. None of the knockout mouse models of C9ORF72 recapitulate the phenotype of MND and FTD (Koppers et al., 2015; Lagier-Tourenne et al., 2013). However, it was proposed that haploinsufficiency of the protein may be associated with toxic gain of function including RNA through the accumulation of toxic dipeptide repeat proteins and the abnormal transcription from repeat-associated non-AUG (RAN) translation (Babic Leko et al., 2019; Balendra and Isaacs, 2018).

The pattern of inheritance for the mutation is autosomal dominant with incomplete penetrance and the age of onset associated with the mutation is ~58 years (Majounie et al., 2012). There is variability of disease phenotype between individual families, reflecting differences in penetrance. C9ORF72 expansions are associated with the diagnosis of FTD (Sha et al., 2012), cerebellar ataxia (Corcia et al., 2016), Huntington’s disease (Hensman Moss et al., 2014) and schizophrenia (Galimberti et al., 2014). Many of these disorders are overlooked during MND assessment of family history. A characteristic of C9ORF72 mutations is the presence of transactive (TAR) DNA-binding protein- 43 (TDP-43) in ubiquitinated neuronal and glial cytoplasmic inclusions (Arai et al., 2006; Neumann et al., 2006).

1.1.4.4 Cu/Zn superoxide dismutase-1 (SOD1)

SOD1 was the first gene to be associated with MND (Rosen et al., 1993), and its discovery accelerated our understanding of MND pathogenesis. The clinical presentation of SOD1 familial cases is very similar to sporadic MND. SOD1 gene is located on chromosome 21 comprising of 5 exons and 4 introns that spans over 11 kilobases of genomic DNA and encodes a 153 amino acid protein that exists as a 32kDa homodimer (Tainer et al., 1982). Each SOD1 monomer forms a subunit with two metal ions, the catalytic copper and stabilizing zinc, to carry out its enzymatic function. SOD1 is a soluble protein that belongs to the family of superoxide
dismutase enzymes. There are three isoforms of superoxide dismutase, each with different structural characteristics. The familial MND associated SOD1 mutations are found throughout the protein (Cleveland and Rothstein, 2001), and almost all are dominantly inherited. Although the exact pathogenic mechanisms underlying SOD1 associated MND is still not well understood, it is believed that a gain of toxic function is responsible for the degradation of neurons since many mutant forms of the SOD1 protein remain catalytically active (Borchelt et al., 1994; Hodges, 2012).

Transgenic mice overexpressing the human mutant forms of Cu/Zn SOD1 phenotypically present symptoms similar to MND patients (Gurney et al., 1994; Wong et al., 1995). Therefore, Cu/Zn SOD1 transgenic mouse models have proven to be a very useful model to investigate the causes, disease progression, pathological features as well as test potential therapeutics treatments for MND.

1.2 SOD1 transgenic models of MND

Disease onset and progression in familial cases of MND vary depending on the type of mutation patient carries. Some mutations show rapid disease progression with severe symptoms and death can occur within 4 years of diagnosis, such as SOD1-G93A mutation. While others show mild symptoms and disease can be manifested much longer, for example, SOD1-H46R mutation with disease duration of up to 17 years (McAlary et al., 2013; Pan et al., 2012). This variability can make human studies difficult for investigating MND at each clinical stage from disease onset to death. As a result, MND research has been relying on animal models with the hope to gain insight into the mechanisms and pathogenesis of the disease that may suggest possible therapeutic approaches.

Currently, the most widely used and well-established animal models of MND are transgenic mice expressing the human mutant forms of SOD1. Currently, more than 12 different SOD1 mutations that have been expressed in transgenic mice. The common SOD1 transgenic mice model used is summarised in Table 1.2, with the most well-studied lines includes SOD1-G93A, SOD1-G37R, and the SOD1-G85R. Transgenic mice over-expressing the mutant forms of SOD1 develop motor neurone disease reminiscent of the phenotypes seen in human MND. This is characterised by limb tremors, limb weakness and eventually complete paralysis at the end stage of the disease (Bruijn et al., 1997; Gurney et al., 1994; Wong et al., 1995).

9
Table 1.2 Commonly used MND mutant SOD1 transgenic mouse models.

<table>
<thead>
<tr>
<th>SOD1 mouse model</th>
<th>Symptoms onset (age weeks)</th>
<th>Survival (age weeks)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>G93A</td>
<td>13 to 17</td>
<td>17 to 26</td>
<td>(Gurney et al., 1994)</td>
</tr>
<tr>
<td>G37R</td>
<td>15 to 17</td>
<td>25 to 29</td>
<td>(Wong et al., 1995)</td>
</tr>
<tr>
<td>G85R</td>
<td>35 to 43</td>
<td>37 to 45</td>
<td>(Bruijn et al., 1997)</td>
</tr>
<tr>
<td>G86R</td>
<td>12-16</td>
<td>~17</td>
<td>(Ripps et al., 1995)</td>
</tr>
<tr>
<td>L84V</td>
<td>21 to 26</td>
<td>26 to 30</td>
<td>(Tobisawa et al., 2003)</td>
</tr>
<tr>
<td>H46R</td>
<td>~20</td>
<td>~24</td>
<td>(Chang-Hong et al., 2005)</td>
</tr>
<tr>
<td>A4V³</td>
<td>~35</td>
<td>~48</td>
<td>(Deng et al., 2006)</td>
</tr>
<tr>
<td>D90A</td>
<td>~52</td>
<td>~61</td>
<td>(Jonsson et al., 2006)</td>
</tr>
<tr>
<td>G127X</td>
<td>~32</td>
<td>33-35</td>
<td>(Jonsson et al., 2004)</td>
</tr>
<tr>
<td>L126X</td>
<td>~44</td>
<td>~37</td>
<td>(Deng et al., 2006)</td>
</tr>
</tbody>
</table>

Transgenic mice expressing the SOD1 mutation show the formation of ubiquitin-positive SOD1 inclusions, a pathological hallmark of familial MND (Tu et al., 1996; Wang et al., 2003; Watanabe et al., 2001; Yang et al., 2014). The amount of inclusions present in these transgenic mice depends on the mutation and the transgene copy number. SOD1 transgenic mice expressing the mutation G37R and G93A exhibit prominent vacuolation in the mitochondria with a lower number of inclusions, while mice expressing the H46R and G85R mutations show higher levels of inclusions (Watanabe et al., 2001; Wong et al., 1995). Mice that express more of the mutated gene are more severely affected (Alexander et al., 2004; Dal Canto and Gurney, 1995). SOD1-G93A mice with 25 copies of the mutant SOD1 gene (Gurney, 1997) have an earlier age of onset (~13-17 weeks of age) and death (17-26 weeks) compared to SOD1-G37R (42) mice with 5-12 copies of the mutant SOD1 gene having a later disease onset at approximate at 15 weeks of age (Dal Canto and Gurney, 1995; Roberts et al., 2014) and death at (25-29 weeks) (Wong et al., 1995).

In this study, the SOD1-G37R transgenic mouse model of MND will be used. The study will take into account the survival times, disease progression, and pathological events manifested in these mice.

1.2.1 SOD1-G37R mouse models

The SOD1-G37R line expresses human SOD1 cDNA with a mutation at amino acid position 37, glycine replaced by arginine (Wong et al., 1995). SOD1-G37R mice express mutant SOD1
proteins at levels 5 to 12 times higher than endogenous SOD with normal enzyme activity. Several variants of this SOD1-G37R mouse model have been developed including line 9, 29, 42, and 106 (Wong et al., 1995). Of these variants line 29 (7 times increase in SOD1 activity) and 42 (14.5 times increase in SOD1 activity) SOD1 expressing variants are typically used (Wong et al., 1995). Both mouse lines develop a severe, progressive, MND resembling the G93A transgenic mice. The pathological features of both lines also reflect what is seen in human cases including neurofilament inclusions, positive ubiquitin aggregates, selective motor neuron loss, astrogliosis and muscle atrophy. SOD1-G37R (42) has a higher gene copy number and they develop earlier clinical signs of MND at approximately 14-16 weeks compared to SOD1-G37R (29) mice that develop symptoms at 26-28 weeks of age (Wong et al., 1995). Both lines developed tremors, progressing to hindlimb paresis, muscle atrophy, reduced grip strength, and locomotor deficits at 25 weeks. Endstage for SOD1-G37R (42) is 27-29 weeks and 32-35 weeks for SOD1-G37R (29) (McAllum et al., 2013; Nguyen et al., 2000).

This study uses SOD1-G37R line 42, due to its shorter disease timeline and better characterised phenotypes compared to line 29. When compared alongside other transgenic SOD1 mice, the SOD1-G37R mouse develops the disease over a longer timeframe. Despite the longer lifespan, SOD1-G37R reliably produces a disease phenotype reminiscent of the other more popular mouse models of MND such as G93A. The longer lifespan allows more time during the presymptomatic stage to measure any modulation of disease onset and progression, especially when a new strain of mouse is developed (SOD1-G37R:APLP2-knockout). Thus, the SOD1-G37R (42) is ideal for the current study, allowing for more flexibility in the experimental design concerning the generation of a new mice strain, and allows sufficient time for mice to mate and reproduce.

1.3 Neuropathology of MND: Proposed mechanisms of motor neurons degeneration

The pathological hallmark of MND is the loss of the motor function, with the degeneration of motor neurons in the ventral horns along the spinal cord, as well as in the brain stem and the motor cortex, but to a lesser extent (Figure 1.2 The disease process of MND). The degeneration of motor neurons is accompanied by astrogliosis affecting the white and grey matter of the brain. There are three types of protein aggregates generally observed in the surviving motor neurons of MND;
i) the skein like inclusions which are usually ubiquitin-positive,

ii) round hyaline that is also ubiquitin positive and

iii) bunina body that is generally ubiquitin negative.

There is also cytoplasmic shrinkage and lipofuscin granules observed in the neurons. Aggregate formations in skein like inclusions are filamentous in structure. The bunina bodies are small eosinophilic inclusions found within the cytoplasm or dendrites (Kato, 2008). The round hyaline inclusions are eosinophilic and usually found localised to motor neurons in the anterior horn of the spinal cord. Within the skein-like and hyaline inclusions, several proteins have been identified including SOD1, TDP-43, and FUS, which will be described later in this thesis. In the bunina bodies there is immunoreactivity to cystatin C and transferrin detected (Okamoto et al., 1993; Okamoto et al., 2008).

Although substantial research has been put forward to understand MND, the actual mechanism(s) responsible is still not yet fully known, and the aetiology of the disease remains to be elucidated. However, the transgenic mouse models have enabled researchers to put forward several hypotheses to explain the pathogenesis of the disease including; protein aggregation, glutamate excitotoxicity, mitochondrial dysfunction, motor unit dysfunction etc.
Figure 1.2 The disease process of MND

MND is a disease of unknown causes, the disease affects the fatality rate of the upper motor neurons in the brain cortex and the lower motor neurons in the motor nuclei of the brain stem and the anterior horn of the spinal cord. These motor neurons are the nerve cells that innervate the voluntary muscles controlling our speech, swallowing and movement. When these motor neurons die the muscles begin to waste away resulting in weakness of the skeletal muscle. The progressive paralysis of the respiratory muscle and the subsequent respiratory dysfunction is the cause of death.

1.3.1 Protein aggregation

The formation of aberrant proteins aggregates and inclusions are common pathological features in many neurodegenerative disorders. The protein aggregates are believed to be derived from the accumulation of misfolded proteins. In MND, the presence of protein aggregates was observed in both sporadic and familial cases, and as well in transgenic mouse models of MND (Bruijn et al., 1997; Gurney et al., 1994; Wong et al., 1995). Several proteins have been identified within the aggregates, including SOD1, TDP43, FUS, OPTN, UBQLN2 and C9ORF72 (Bruijn et al., 1998; Bruijn et al., 2004; Deng et al., 2011a; Deng et al., 2011b; Deng et al., 2010; Keller et al., 2012; Neumann et al., 2006; Wong et al., 1995).

1.3.1.1 SOD1 aggregation and toxicity

Apart from motor neurons, SOD1 aggregates are found in astrocytes and glial cells in all cases of MND, and is not restricted to SOD1 mutations (Bosco et al., 2010; Pare et al., 2018),...
suggesting that there is a SOD1 dependent glial component. It is unknown whether these astrocytic changes are a secondary reaction to the degenerating neurons or are primary toxic agents or a combination of both.

It was postulated that the toxicity of mutated SOD1 was the result of the loss of enzyme function. However, this theory was overturned when SOD1 KO mice did not develop a motor neuron degeneration phenotype, but instead developed liver malignancies and a shorter life span (Fischer et al., 2011; Fischer et al., 2012; Reaume et al., 1996). Furthermore, expressing mutant SOD1 transgene in a SOD1-KO background did not alter the severity of disease symptoms or disease onset (Bruijn et al., 1998; Jaarsma et al., 2000). In almost all cases the mutant SOD1 has catalytic activity similar to WT-SOD1 (Bosco et al., 2010). Transgenic mouse models carrying one mutant allele as well as those that over-expressing the human mutant forms of SOD1 develop MND like phenotypes (Reaume et al., 1996). This suggests that a mutant SOD1 gene causes dominant inheritance with acquired neurotoxic function(s) and is independent of SOD1 activity levels.

How SOD1 accumulates and form aggregates and whether aggregates themselves are the neurotoxic cause of the disease is not known. Several hypotheses have been proposed for the toxicity to be due to SOD1 protein aggregates, which includes the insufficient zinc protein binding or impaired protein degradation hypotheses. In the zinc binding hypothesis, it is proposed that zinc bind less effectively to mutant SOD1 compared to the WT-SOD1. Mutant SOD1 with zinc depletion leads to rapid neuronal death with elevated levels of nitrotyrosine (Crow et al., 1997a; Crow et al., 1997b). This hypothesis is still controversial since it is still unclear how 150 different mutations can manipulate the binding of zinc. Furthermore, beneficial phenotypes were observed in SOD1 transgenic mice with nitric oxide deletion (Facchinetti et al., 1999) which suggests zinc depletion does not cause the pathogenesis seen in SOD1 transgenic mice. Also, in transgenic mice with G37R or G85R mutation tyrosine nitration was not observed, which further supports that the reduction of zinc is not the cause of SOD1 mutation (Bruijn et al., 1997; Williamson et al., 2000).

While dysfunction in the ubiquitin protein proteasome system is the main basis underpinning the protein degradation hypothesis. Accumulation of ubiquitinated protein inclusions has been found in both familial and sporadic cases of SOD1 mediated MND (Ince et al., 1998; Wang et al., 2003; Watanabe et al., 2001). Under normal conditions, ubiquitin is responsible for protein
removal through the proteasome system. In many cases of MND proteasome dysfunction have been observed, and non-neuronal cells expressing mutant SOD1 show proteasome inhibition after protein aggregate formation (Kabashi et al., 2004; Puttaparthi and Elliott, 2005). This suggests proteasome malfunction could be a primary cause of protein aggregation in SOD1-mediated MND.

1.3.1.2 Transactive response DNA binding protein

Transactive response (TAR) DNA-binding protein- 43 (TDP-43) is a nuclear protein found in ubiquitinated inclusions in FTLD and MND patients (Arai et al., 2006; Neumann et al., 2006). Mutated TDP-43 is cleaved and abnormally phosphorylated and accumulates into ubiquitinated cytoplasmic inclusions in motor neurons (Chen-Plotkin et al., 2010). TDP43 inclusions are found in ~98% of all cases of MND (Neumann et al., 2006) residing in spinal cord motor neurons, hippocampal and frontal cortex neurons, and glial cells of all sporadic cases as well as the majority of familial patients that are SOD1 mutant negative (Mackenzie et al., 2007; Tan et al., 2007). Mutations in TDP-43 are mainly located in the C-terminus of the protein and contribute to approximately 5% of familial MND and approximately 1% of sporadic cases (Millecamps et al., 2010). TDP-43 predominantly localises to the nucleus and is primarily involved in RNA metabolism (both mRNA and miRNA) including alternative slicing, RNA transport and RNA stability (Scotter et al., 2015). Toxicity was observed when wildtype TDP-43 is overexpressed in yeast and in primary cultured neurons, and this is more pronounced with overexpressed mutant TDP-43 (Barmada et al., 2010; Fallini et al., 2012; Johnson et al., 2008; Kabashi et al., 2010). The toxic effect of TDP-43 is dose dependent (Swarup et al., 2011; Tsai et al., 2010; Turner et al., 2008; Wils et al., 2010). The overexpression of wildtype TDP-43 in transgenic mice leads to neuronal degeneration and causes spastic quadriplegia (Wils et al., 2010). Transgenic mice overexpressing wild-type or mutant forms of TDP-43 developed learning, memory and motor deficits, and had a reduced lifespan (Tsai et al., 2010; Tsuiji et al., 2017; Turner et al., 2008; Wils et al., 2010). Mutant TARDBP are exclusive to MND and not observed in other neurodegenerative disorders (Rutherford et al., 2008; Van Deerlin et al., 2008), except in a few cases of FTD (Benajiba et al., 2009; Borroni et al., 2009; Kovacs et al., 2009; Rutherford et al., 2008; Van Deerlin et al., 2008).

1.3.1.3 Fused in Sarcoma (FUS)

The discovery of TDP-43 urged researchers to examine other RNA binding protein, which included Fused in sarcoma (FUS) or translocated in lymphosarcoma (TLS) in familial MND
patients (Kwiatkowski et al., 2009). FUS mutations account for ~4% of familial and ~1% of sporadic MND cases (Kwiatkowski et al., 2009; Vance et al., 2009). FUS is a ubiquitously expressed nuclear RNA binding protein and is involved in the processing of mRNA, splicing and aids in DNA repair (Hoell et al., 2011; Vance et al., 2009). Post-mortem histological examination of FUS mutation carriers showed FUS positive cytoplasmic inclusions in the lower motor neurons with normal TDP-43 expression, suggesting distinguishing features for FUS mutations from other cases of MND (Groen et al., 2010; Hewitt et al., 2010; Vance et al., 2009). In addition, disease progression in juvenile cases of FUS mutation carriers is rapid without the development of FTD and displays a distinct pathology marked by neuronal basophilic inclusion that are immunoreactive for FUS protein (Abramov and Duchen, 2008; Baumer et al., 2010). The patterns of FUS positive immunoreactivity in MND cases without FUS mutation is still not understood. Studies have reported that FUS is not present in sporadic MND patients nor familial SOD1 mutant patients (Huang et al., 2010; Neumann et al., 2009; Vance et al., 2009). In contrast, some studies have reported that FUS positive inclusions with signals for TDP-43, p62 and ubiquitin observed in all sporadic and familial MND cases except for those that are mutant SOD1 carriers (Deng et al., 2010; Keller et al., 2012). FUS protein is enriched in the soluble fraction of the brain from subjects with frontotemporal lobar degeneration (FTLD) and FUS pathology (Neumann et al., 2009). In addition, FUS aggregates occur in other neurodegenerative disorders including Huntington’s disease and spinocerebellar ataxia suggesting that the disruption of endogenous FUS metabolism is associated with other neurodegenerative processes (Doi et al., 2010; Neumann et al., 2009).

1.3.1.4 Optineurin (OPTN)
Optineurin (OPTN) is a rare genetic variant in MND and has been reported in Japanese patients from consanguineous marriage (Maruyama et al., 2010). In sporadic MND, OPTN is found in cytoplasmic skein like aggregates and colocalise with ubiquitin, TDP43 and FUS (Deng et al., 2011a; Hortobagyi et al., 2011; Keller et al., 2012; Maruyama et al., 2010). Confounding results were observed in studies for OPTN. Immunoreactive OPTN colocalised to SOD1 positive aggregates in mutant SOD1 carriers. In contrast, other studies observed OPTN in both sporadic and non-mutant SOD1 familial patients (Keller et al., 2012; Maruyama et al., 2010), and were not present in SOD1 transgenic models (Deng et al., 2011a; Hortobagyi et al., 2011). Likewise, positive OPTN immunoreactivity occurred in FUS carriers (Ito et al., 2011; Keller et al., 2012) while others reported OPTN was not detected (Hortobagyi et al., 2011). OPTN is involved in
NFκB signalling and regulating vesicular and endoplasmic trafficking (Vaibhava et al., 2012). Similar to other aggregates, OPTN is also present in other neurogenerative diseases including dementia with MND, Huntington’s disease, Alzheimer’s disease, Parkinson’s disease, Creutzfeldt-Jacob disease, multiple system atrophy and Pick’s disease (Osawa et al., 2011).

1.3.1.5 Ubiquilin-2 (UBQLN2)
Ubiquilin-2 (UBQLN2) has been identified to cause a dominant X-linked familial form of MND (Deng et al., 2011b). Post mortem histological examination of the spinal cord of UBQLN2 mutation carriers showed positive immunoreactivity for UBQLN2 in skein like inclusions (Deng et al., 2011b; Williams et al., 2012). UBQLN2 was present in the hippocampus in dementia cases of MND and absent in dementia cases without MND, suggesting the presence of UBQLN2 aggregates are linked to a dementia phenotype (Deng et al., 2011b). The exact function of UBQLN2 is unknown, but it is known to be involved in the degradation of ubiquitinated proteins by the ubiquitin proteasome system (UPS) and autophagy (Deng et al., 2011b). Overexpression of mutant UBQLN2 causes dysfunction of the UPS (Lee and Brown, 2012) by an unknown mechanism.

1.3.2 Glutamate toxicity
Another theory to explain the cause of MND pathology is glutamate excitotoxicity. The cerebrospinal fluid of MND patients has an increase in glutamate levels (Rothstein et al., 1990; Shaw and Ince, 1997). In normal neurons, the process for stimulating glutamate clearance is tightly regulated at the synaptic cleft. If glutamate clearance fails this results in overstimulation and hyperexcitability in motor neurons, resulting in cell death due to excessive calcium influx and increased oxidative stress (Abramov and Duchen, 2008). From this observation the anti-glutaminergic drug Riluzole was put forward as an MND treatment (Bensimon et al., 1994). Riluzole is the first approved FDA-drug to decrease the levels of glutamate toxicity (Bensimon et al., 1994; Lacomblez et al., 1996). However, the lifespan of MND patients is only extended on average by a few months with Riluzole treatment (Turner and Talbot, 2008).

1.3.3 Mitochondrial dysfunction
In the motor neurons and muscles of MND patients and transgenic SOD1 mice, there is evidence of mitochondria dysfunction, including vacuolization and swelling (Borthwick et al., 1999; Wiedemann et al., 1998). This suggests mitochondria are a primary target of SOD1-mediated toxicity. Mitochondria play several roles in cells including energy production, and
the by-product of this process is reactive species that can cause oxidative stress. Several hypotheses were proposed involving defects in the import of mitochondrial proteins and disrupted calcium homeostasis (Damiano et al., 2006; Kawamata and Manfredi, 2010; Kong and Xu, 1998; Mattiazi et al., 2002). While the observed glutamate excitotoxicity may be a secondary cause, the mechanisms of mitochondrial toxicity in MND and how it interacts with other pathological events in MND remains unknown.

1.4 Motor unit dysfunction in MND and ageing
It is proposed that MND is a multifactorial and multisystemic disease, which may explain the difficulties in developing therapeutics. A key issue is identifying the triggering site that causes the progressive degeneration of the upper and lower motor neurons and subsequently muscle atrophy. This remains an ongoing debate and an important question is which structure of the motor unit is affected first? - the motor neurons, neuromuscular junction or muscles?

As highlighted in Section 1.1.3, ageing is one of the major risk factors of MND. Natural ageing and age-related neurodegenerative diseases both lead to neuronal and synaptic dysfunction. It was once thought that the functional deficits observed in ageing are in part due to neuronal death (Mattson and Magnus, 2006). However, it has now become clear that synaptic dysfunction plays a major role in normal ageing with very low neuronal death (Burke and Barnes, 2006; Duan et al., 2003; Saxena and Caroni, 2011; von Bohlen und Halbach et al., 2006). In contrast, neuronal cell death plays a major role in neurodegenerative diseases, a prominent feature of MND (Nijssen et al., 2017). Nonetheless, synaptic terminal dysfunction is a process that occurs both in normal ageing and MND, resulting in muscle weaknesses, wasting and a decline in motor function (Campanari et al., 2016; Cappello and Francolini, 2017; Courtney and Steinbach, 1981; Deschenes et al., 2010; Fischer et al., 2004; Luff, 1998). Since motor decline in ageing is a shared common feature with other neurodegenerative processes (Valdez et al., 2012), it is important to understand if this process is altered in MND. In the subsequent sections we will discuss the structures of a motor unit and their associated function in MND pathogenesis and ageing.

1.4.1 Structure of a motor unit
Skeletal muscle activities are under the control of the motor unit, regulated by signals from the motor neuron (Monti et al., 2001). A motor unit comprises of single motor neuron and all the muscle fibres it innervates. As the motor axons reach their target muscle they are separated into
terminal branches and form synapses with the muscle fibre. This highly specialised region is called the neuromuscular junction (NMJ) (Hall and Sanes, 1993).

### 1.4.1.1 Motor neurons

Motor neurons are the only neurons of the CNS with cells bodies located in the anterior horn of the spinal cord and axons that extend to innervate non-neuronal tissues (Stifani, 2014). There are two main types of motor neurons: the upper motor neurons and the lower motor neurons. The upper motor neurons originate from the motor cortex of the brain and their axons project into the spinal cord. While the cell bodies of the lower motor neurons lie in the spinal cord and project their axons to the periphery innervating muscles (Stifani, 2014). Motor neurons that innervate the same group of muscle fibres are clustered together into the motor nuclei and they tend to extend over several spinal cord segments (Burke et al., 1977; Hollyday et al., 1977; Landmesser, 1978). In the lumbar and cervical spinal cord segments, the motor neurons that innervate the distal musculature are located laterally, and more medially for the proximal musculature within the anterior horn region of the spinal cord (Burke et al., 1977).

In mammals there are three main classes of motor neurons in the motor nucleus; alpha motor neurons, gamma motor neurons and beta motor neurons (Manuel and Zytnicki, 2011). These neurons differ in the muscle fibres they innervate (i.e. differ in function), molecular properties, electrical properties and their susceptibility to degeneration. The α motor neurons have large cells bodies and they innervate the extrafusal muscle fibres which comprise the bulk of the skeletal muscle tissue and are responsible for generating tension via muscle contraction (Burke et al., 1977; Eccles et al., 1960). The γ motor neurons are smaller in size compared to the α motor neurons and they innervate the intrafusal muscle fibres (Eccles et al., 1960). The intrafusal muscle fibres consist of the muscle spindle innervated by two axons; sensory involved in proprioception and motor controlling muscle tension (Manuel and Zytnicki, 2011). The third types of neurons are the β motor neurons and they are generally found in higher primates, and possibly in humans. β motor neurons are sometimes called skeleton-fusimotor, and they innervate both the intrafusal and extrafusal muscle fibres (Bessou et al., 1965). β motor neurons are difficult to identify, therefore little is known about their physiological roles (and why their presence in humans is unclear).

Among the three classes of motor neuron pools, α motor neurons are the most abundant and they can be classified into three subtypes based on their contractile properties; fast-twitch
fatigable (FF), fast-twitch fatigue resistant (FFR), and slow twitch fatigue resistant (SFR) (Burke et al., 1977). These α motor neurons differ in the size, excitability and firing patterns, and conduction velocity. SFR has a smaller cell body calibre and a higher input resistance, therefore they are responsive to lower stimulation threshold. During normal muscle contraction SFR motor neurons are recruited first (Mendell, 2005). They have the capacity to maintain activity even when the stimulation has ceased (Lee and Heckman, 1998).

In contrast, FF motor neurons have a larger cell body calibre and they are recruited after SFR neurons (Mendell, 2005). In terms of conduction velocities, SFR has a slower (85 m/sec) conduction velocity compared to FF (100 m/sec) (Burke et al., 1977). Little is known about the physiological function of FFR, but they are thought to have intermediate characteristics between SFR and FF motor neurons (Burke et al., 1977; Lee and Heckman, 1998).

Although motor neurons receive and relay all motor commands to muscles, the effect of ageing on the survival of these neurons is still an ongoing debate. Many studies have reported a reduction in α motor neurons in aged humans and animals, and they showed that hypertrophy was observed in the remaining α neurons (Jacob, 1998; Tomlinson and Irving, 1977). In contrast, there are studies reporting no difference regarding the number and size of motor neurons in aged mice compared to adult mice (Chai et al., 2011; Maxwell et al., 2018).

In MND, not all motor neurons are susceptible to degeneration (Hadzipasic et al., 2014; Kaplan et al., 2014). Oculomotor and Onuf’s nuclei neurons are spared in MND even at the end stage of disease (Iwata and Hirano, 1978; Kubota et al., 2000; Mannen et al., 1982). While the α motor neuron pool in the spinal cord progressively and selectively degenerate as the disease progresses (Conradi and Ronnevi, 1993; Hadzipasic et al., 2014; Lalancette-Hebert et al., 2016). The factors contributing to the selective vulnerability in distinct motor neurons pools in MND is unknown. It is speculated that the size, morphology and organisation of motor pools, as well as synaptic transmission, plays a major role in this selective vulnerability (Campanari et al., 2016; Cappello and Francolini, 2017; Fischer et al., 2004; Lalancette-Hebert et al., 2016; Nijssen et al., 2017).

1.4.1.2 Neuromuscular junction

The neuromuscular junction (NMJ) is the site of communication between motor nerve axons and skeletal muscle fibres (Li et al., 2018). The synaptic junction functions to transmit signals
or nerve impulses via the neurotransmitter acetylcholine (Ach) from the axonal terminus to the muscle fibres, thereby initiating muscle contraction (Li et al., 2018). Although very few neurons die during ageing, the reduction in the number of motor neurons in the spinal cord is seen in ageing rats (~36 months old) (Rowan et al., 2012). The reduction in muscle mass and motor neuron numbers with ageing are proposed to be associated with NMJ remodelling, which precedes muscle fibre atrophy and could initiate muscle loss (Arnold et al., 2014; Deschenes et al., 2010; Mantilla and Sieck, 2011). Interestingly, muscles separated from their NMJ show no evidence of atrophy suggesting that local targets (e.g. spinal cord) rather than upstream factors might be responsible (Mantilla and Sieck, 2011; Prakash and Sieck, 1998). NMJ morphology changes during ageing have been postulated to be remarkably similar to the naturally occurring process of synapse elimination that occurs during development and in response to nerve injury (Apel et al., 2009; Arnold et al., 2014; Balice-Gordon, 1997).

Ageing has been associated with morphological changes that were restricted to nerve terminals with little or no degeneration of axons, suggesting that ageing was associated with functional synaptic denervation (Fujisawa, 1976; Gutmann and Hanzlikova, 1972). Aged nerve terminal endings release larger amounts of neurotransmitter stimulation, resulting in higher endplate potential at the muscle fibre (Deschenes et al., 2011). This may seem to be a compensatory mechanism to improve NMJ transmission, but this results in a greater rate of NMJ dysfunction that is often seen in aged animals (Mantilla and Sieck, 2011).

NMJ denervation is characterised by retraction of nerve axons terminal resulting in the loss of specific synapses between nerve axons branches and postsynaptic receptors (Deschenes et al., 2010). During NMJ denervation the neighbouring motor axons compensate for the loss of the synaptic junction by sprouting new branches and reinnervating the previously denervated muscle fibres (Jang and Van Remmen, 2011). This compensatory mechanism is dramatically diminished in ageing animals (>18months) (Balice-Gordon, 1997). When the denervation process exceeds the compensatory capabilities of reinnervation, this leads to the elimination of denervated muscle fibres, and the loss of muscle mass becomes apparent in aged animals (~24-36 months) (Balice-Gordon, 1997). Newly formed junctions are markedly vulnerable to degenerative changes, and some of these junctions disappear within weeks. C57BL/6 mice at 33 month old, compared to 11 months old mice, have more than two-thirds of NMJs either partially innervated or completely denervated with extensive sprouting of motor neurons (Jang
and Van Remmen, 2011). The same study reported that muscle atrophy and synaptic changes in the ageing muscles occur in a fibre by fibre manner, suggesting that denervation changes at the NMJ are key factors in skeletal muscle atrophy during ageing. NMJs were observed to remain stable through lifespan and only become fragmented during regeneration that occurs following injury to muscle fibres. This suggested NMJ remodelling occurs prior to muscle fibre atrophy (Li et al., 2011). Similar to ageing, NMJ denervation is one of the earliest events that occur in MND, and it occurs prior to motor neuron degeneration and muscle atrophy (Fischer et al., 2004).

Despite the numerous studies on the mechanisms behind motor neuron degeneration in MND, the precise location that initiates motor neuron degeneration is still controversial. Three hypotheses have been proposed to illustrate this process: the dying forward (Eisen and Weber, 2001), the dying backward (Dadon-Nachum et al., 2011) and dying outwards (Baker, 2014). The dying forward hypothesis proposed that motor neurons in the cortex are the primary site that initiates this process and it then extends in an anterograde manner to corticospinal projections (Eisen and Weber, 2001). In the dying backward hypothesis, it was proposed that the disease is a distal axonopathy in which the primary site that initiates motor neuron degeneration begins at the nerve terminals and progress towards the cell bodies in a dying back manner (Dadon-Nachum et al., 2011). While in the dying outward explanation this integrates both the “dying forward” and “dying backward” hypothesis (Baker, 2014).

Given the complexity of the disease it is highly possible that both the dying backward and dying forward processes can occur independently from each other. Nevertheless, it is accepted that NMJ dismantling results in muscle atrophy, a key factor in MND symptoms onset and pathogenesis. This was evidenced in SOD1-G93A mice studies where distal axonal and NMJ changes were altered prior to symptom onset (Clark et al., 2016). MRI and electrophysiology studies in these mice also suggest that NMJ degeneration precedes motor neuron loss (Brooks et al., 2004; Dobrowolny et al., 2008; Hegedus et al., 2007; Wong and Martin, 2010). It is clear that the loss of muscle mass, reduction of spinal motor neurons number and NMJ denervation are common features observed both in ageing and in MND patients.

1.4.1.3 Muscle fibres
Muscle atrophy as a result of motor neuron loss and NMJ denervation is a common feature in MND. During NMJ remodelling in ageing and injury, when a motor neuron innervating a
muscle bundle dies the neighbouring axons will reinnervate those muscle fibres and exhibit NMJ remodelling by a non-random regrouping of muscle fibre type (Jang and Van Remmen, 2011). While this compensatory mechanism is dramatically decreased in ageing (Rudolf et al., 2016), in MND it has been suggested that axon sprouting is unable to change the fibre types of the reinnervated muscle (Baloh et al., 2007; Daube, 2000). The reduction of muscle mass in ageing is most prominent in the lower limbs, and is accompanied by a 25-30% decrease of skeletal muscle fibres cross sectional area, 30-40% decrease in muscle fibre number and a 30-40% decrease in muscle strength (Porter et al., 1995; Vandervoort, 2002). While no significant changes in fibre type distribution was found, there is a reduction in muscle fibre size for the smaller type II fibre, but an increase in the diameter of the larger type II fibres that are mainly type IIa fibres (Frontera et al., 2008). Type I muscle fibres are not affected (Frontera et al., 2008; Janssen et al., 2000; Verdijk et al., 2007), and can be explained by denervation of motor units of type II fibres and the collateral reinnervation of type I fibres in ageing (D'Antona et al., 2003).

Ageing is also associated with transformation of muscle fibre type from fast to slow fibre and affects mostly type IIx. These changes are associated with age-related changes at the motor unit (Ciciliot et al., 2013). However, it is important to highlight that the reduction in muscle fibre size is not an explanation for the loss of muscle mass and strength in ageing. It is in part largely due to the force generating capacity by skeletal muscle (D'Antona et al., 2003; Lowe et al., 2002; Yu et al., 2007) and several other factors including mitochondrial dysfunction (Broskey et al., 2014; Joseph et al., 2013), myofilament alterations (D'Antona et al., 2003; Frontera et al., 2008; Lowe et al., 2002), uncoupling of the excitation and contraction process (Renganathan et al., 1997), and adipocyte infiltration (Goodpaster et al., 2000).

Although little is known about the role of muscle integrity in preserving fibre innervation in MND, it has been suggested that mutant SOD1 induces a toxic effect on the muscle leading to the impairment of mitochondrial activity in muscles (Da Cruz et al., 2012). It is still debated whether muscles play a role in promoting NMJ denervation and motor neuron degeneration. While muscle specific expression of mutant SOD1 was shown to induce a reduction in muscle mass and strength and mitochondrial dysfunction, motor neuron numbers were not changed (Lino et al., 2002; Pramatarova et al., 2001). In contrast, another study in transgenic SOD1 mice exhibited pathology reminiscent of MND, including NMJ denervation and motor neuron
degeneration (Wong and Martin, 2010). Regardless whether muscle fibres play a role in inducing the pathological events in MND, undoubtedly, significant morphological changes are observed in the muscle fibres of MND patients, revealing significant atrophy and compensatory hypertrophy of both type I and type II fibres (Jensen et al., 2016). Similar observations were observed in SOD1 transgenic mice (Hegedus et al., 2008; Julien and Kriz, 2006). While the direct contribution of muscles to the degenerative process of MND remains controversial, these data suggest possible overlap and cross-talk among the three building blocks of the motor unit (motor neurons, NMJ and muscle fibres) in the molecular interaction between ageing and MND.
1.5 The Amyloid Precursor Protein Family

Alzheimer’s disease (AD) is a neurodegenerative disorder of the central nervous system (CNS) characterised by neuronal loss and the deposition of aggregated neurotoxic forms of amyloid β in the brain of patients and neurofibrillary tangles. Amyloid beta is typically described as a 39-43 amino acid peptide (Glenner and Wong, 1984; Masters et al., 1985a; Masters et al., 1985b), derived from the proteolytic processing of the 695-770 amino acid amyloid precursor protein (APP) (Goldgaber et al., 1987; Kang et al., 1987; Tanzi et al., 1987). APP has been primarily studied due to its central association with AD. APP is part of a larger multigene family consisting of at least 16 orthologues with similar domains and structures (Coulson et al., 2000). The two mammalian APP paralogues are amyloid precursor like-protein 1 (APLP1) and amyloid precursor like-protein 2 (APLP2) (Wasco et al., 1992; Wasco et al., 1993). Non-mammalian APP homologues also exist such as apl-1 in \textit{C.elegans}, APPL in \textit{D. melanogaster} and app/appb in \textit{D. rerio} (Daigle and Li, 1993; Rosen et al., 1989). APP family members are typically type 1 transmembrane cell-surface glycoprotein, with a single membrane-spanning domain containing the large ectoplasmic N terminal region and a smaller cytoplasmic C terminal region (Kang et al., 1987). They undergo similar proteolytic processing by the secretases resulting in secreted and membrane associated fragments, but neither APLP1 nor APLP2 contains that amyloid β sequence (Eggert et al., 2004; Scheinfeld et al., 2002). These homologues are highly related in sequence. APP shares approximately 42% amino acid sequence homology with APLP1 and about 52% amino acid sequence homology with APLP2. APP also share a higher similarity in its gene structure with APLP2 than APLP1 (Wasco et al., 1993). The structure, proteolytic processing and functions of the APP family will be discussed below.

1.5.1 Functional Domains of APP, APLP1 and APLP2

1.5.1.1 Amyloid Precursor Protein

APP is encoded by a gene localized on chromosome 21 and contain at least 19 exons (Kang et al., 1987) and it undergoes alternative splicing that give rise to numerous isoforms ranging from 365 to 770 amino acids (Sandbrink et al., 1994a). There are three major isoforms of APP that are commonly expressed; APP695, APP751 and APP770. The protein is 110-130kDa in size and was described as a cell surface receptor (Kang et al., 1987). APP can be divided into multiple distinct domains; the ectodomain containing the conserved E1, acidic domain (Ac)
and E2 domain. The E1 domain is subdivided into the HBD/GFLD (heparin binding/ growth factor like domain) (Small et al., 1994) and the CuBD (copper binding domain) (Hesse et al., 1994). The acidic region rich in aspartic acid and glutamic acid (Kang et al., 1987). The Kunitz protease inhibitor (KPI) domain is only present in APP751 and APP770, while only APP770 contains the OX-2 domain (Kitaguchi et al., 1988; Oltersdorf et al., 1989; Tanzi et al., 1988). APP695, APP751 and APP770 all contain the amyloid β (Aβ) sequence. The Aβ fragment is typically described as being between 39-43 amino acids in length, with 28 amino acids in the extracellular domain and 11-15 amino acids from the transmembrane portion (El-Agnaf et al., 2000; Glenner and Wong, 1984; Masters et al., 1985a). The cytoplasmic domain of APP protein consists of the APP intracellular domain (AICD), containing the YENPTY sequence which is conserved in all APP family members (Haass et al., 1992) (Figure 1.3).

1.5.1.2 Amyloid Precursor – Like Protein 1
Amyloid precursor–like protein-1 (APLP1) is the first APP homologue of APP described (Wasco et al., 1993), consisting of 650 amino acids. APLP1 gene is localised to chromosome 19q13.1 (Wasco et al., 1993) with 17 exons (Zhong et al., 1996). APLP1 protein expression is mainly localized to the cell surface while APP and APLP2 are mostly found inside the cell (Kaden et al., 2009). Similar to APP and APLP2, APLP1 protein structure consists of the conserved E1 and E2 domains that are linked by the Ac domain. Unlike APP and APLP2, APLP1 does not contain the KPI domain (Müller et al., 2017). The E2 domain of APLP1 shared a conserved mode of heparin dimerization with APP (Lee et al., 2011; Xue et al., 2011). Only one isoform of APLP1 has been reported (Paliga et al., 1997; Wasco et al., 1992). Similar to APP, APLP1 also contains an intracellular domain termed APLP1 intracellular domain (AL1ICD).

1.5.1.3 Amyloid Precursor – Like Protein 2
Amyloid precursor–like protein-1 (APLP2) gene is localized to chromosome 11 (von der Kammer et al., 1994). It expresses four mRNAs derived by alternative splicing. The main difference between its isoforms is the absence or presence of KPI (exon 15) and the chondroitin sulfate glycosaminoglycan (CS-GAG) site (exon 14) (Thinakaran and Sisodia, 1994; Thinakaran et al., 1995; Wasco et al., 1993). The major isoforms derived from the alternate spicing of APLP2 are 763 and 707 amino acids in length (Sandbrink et al., 1994b; Wasco et al., 1992; Wasco et al., 1993), containing the KPI domain linked by the Ac domain. The remaining isoforms are 751 and 695 which are modified with the addition of CS-GAG at
a single serine residue in the ectodomain (Thinakaran and Sisodia, 1994). While APLP2 763 and 707 contain an additional 12 amino acid insert which abolishes the CS-GAG modification (Wasco et al., 1993).

APLP2 also contains the HBD and CuBD regions, and the structural framework between them is well conserved between APP and APLP2. APLP2 is similar to APP and can bind heparin sulfate via their E1 and E2 domains. In contrast, APLP1 only has one binding site in the E2 domain which has functional relationships with heparin and copper binding activities (Cappai et al., 2005; Dahms et al., 2010). Structural analysis of the APLP2 E2 metal binding sites revealed that it binds to zinc and copper in a similar manner to APP and APLP1 (Roisman et al., 2019). APLP2 also contains an intracellular domain (AL2ICD) that is similar to AICD and it enables them to form dimers to promote intracellular adhesion (Soba et al., 2005; Wasco et al., 1993).

1.5.2 Expression of APP, APLP1 and APLP2

APP is ubiquitously expressed in both neuronal (brain and spinal cord) and non-neuronal tissues (muscles, kidney, lung, pancreas, thymus, spleen, intestine and prostate gland etc.) (Puig and Combs, 2013). APP695 is the major isoform expressed in neuronal tissues (Kang et al., 1987) while APP693 is predominantly found in the liver and fetal tissue and both APP 770 and APP751 are mostly expressed in non neuronal tissues (de Silva et al., 1997; Ohyagi et al., 1990; Sisodia et al., 1993).

Similar to APP, APLP2 expression is highly expressed in the brain, heart, and kidney with lower expression found in the liver and thymus (Wasco et al., 1993). The pattern of APLP2 expression within various brain regions such as the cerebral cortex, hippocampus, thalamus are similar to APP (Wasco et al., 1993). Interestingly, APLP2 expression is highly increased in the cerebellum of AD patients, but APP levels are decreased. This suggests APLP2 expression may be a compensatory response to the reduction in APP expression (Wasco et al., 1992; Wasco et al., 1993). Contrary to APP, only APLP2 is expressed in the small intestine and lungs, and the KPI-containing APLP2 species are found in abundant levels in both neuronal and non-neuronal tissues (Sandbrink et al., 1996; Sisodia et al., 1996; Wasco et al., 1993). APP and APLP2 expression are developmentally regulated and there is a strong increase in its expression during
synaptogenesis (Hung et al., 1992; Löffler and Huber, 1992; Moya et al., 1994). This suggests both APP and APLP2 are important in synapse formation and maturation.

Both APP and APLP2 are expressed in muscles and neurons. In contrast to APP and APLP2, APLP1 is more restricted in its pattern of expression, and is found to primarily in the CNS and its expression is absent in muscles (Klevanski et al., 2014; Slunt et al., 1994). APLP1 expression levels peak during early embryonic development, supporting its role in neurogenesis (Lorent et al., 1995). The distribution and sequence homology between APP and its family members (in particular APLP2) suggests both common but also disparate roles amongst the APP-family members during development, injury or homeostasis.

1.5.3 Proteolytic processing of APP, APLP1 and APLP2

APP undergoes multiple post-translational modifications including glycosylation, sulfation, phosphorylation and palmitoylation as it transits from the endoplasmic reticulum (ER) to the Golgi and through to the plasma membrane. When APP reaches the cell membrane, it is then internalised due to the YENPTY sequence in the C-terminus of APP by clathrin-mediated endocytosis through the endosomal-lysosomal pathway (Haass et al., 1992). The majority of APP is delivered to the lysosome from the endosomes, where it can be degraded and a portion is then trafficked back to the cell surface (Cirrito et al., 2008; Collin and Martens, 2005) to undergo proteolytic processing.

The processing of APLP1 and APLP2 is not as well understood as for APP. It is believed that both APLP1 and APLP2 are processed through similar secretory proteolytic pathways as APP. However, differences can be observed for example; APLP1 is the only member of the family where the processing pathways can be influenced by N-glycosylation (Eggert et al., 2004). Therefore, in this report, I will discuss in detail the processing pathways of APP and compare the differences and similarities observed to APLP1 and APLP2.

The proteolytic metabolism of APP can occur via two major processing pathways: the amyloidogenic pathway and the non-amyloidogenic pathway, where it is cleaved by three types of proteases: α-, β- and γ-secretases (Figure 1.3).
Figure 1.3 Domain structure of mammalian APP family members and proteolytic processing pathways of APP.

APP family members (APP, APLP1, APLP2) share conserved extracellular E1 domains (consisting of HBD and CuBD), Ac and E2 domains. They also contain related APP intracellular domains (ACID) or the APP-like intracellular domain (ALICD) 1 and 2. There are three main neuronal isoforms of APP: APP 695, APP 751 and APP 770. The Aβ region is exclusively found only in APP. Only APP 770 contain Ox2, APP 695 lacks both KPI and Ox2. APP processing pathways consisting of the α-cleavage and β-cleavage. β-cleavage by β-secretase in APP leads to amyloid β production implicated in Alzheimer’s Disease. While during normal cell function APP undergo α-cleavage by α-secretase and γ-secretase to generate p3 fragment. HBD: Heparin binding domain, CuBD: copper binding domain, KPI: Kunitz-type protease inhibitor domain, Ac: acidic domain, Ox2: Ox2 domain, AICD: APP intracellular domain, ALICD: APP like 1 or 2 intracellular domain. Image adapted from (Müller et al., 2017).
1.5.3.1 The β-secretase: the amyloidogenic pathway

The amyloidogenic generates the amyloid-β peptide that is central to AD. APP is first cleaved by β-secretase (also known as BACE1, the β-secretase APP cleaving enzyme 1) at the N-terminus (Vassar et al., 1999) of the amyloid-β domain to release the soluble fragment sAPPβ. The resulting membrane bound C-terminal fragment (CTF-β, also called C99) can be further cleaved by γ-secretase to release AICD and the amyloid-β peptide.

BACE (β site APP cleaving enzyme) is a type 1 transmembrane aspartyl protease expressed in most tissues and is a major neuronal enzyme. There are two highly homologous aspartyl proteases of BACE identified (BACE1 and BACE2) as having the β secretase activity. However, BACE2 is mainly expressed in non-neuronal cells, thereby the cleavage of APP via this secretase within the Aβ domain does not lead to the production of amyloid (Farzan et al., 2000). Apart from APP, other BACE1 substrates include APLP1, APLP2, low density lipoprotein receptor-related protein 1 and Interleukin-1 receptor II (Cai et al., 2012; Hogl et al., 2011; Klaver et al., 2010; Kuhn et al., 2010). Deficiency of BACE1 activity can efficiently block the levels of Aβ secretion (Dominguez et al., 2005; Luo et al., 2001; Roberds et al., 2001). Therefore, BACE1 has become an attractive target for the treatment of AD. When BACE1 is overexpressed in HEK293 cells the processing of APLP1 and APLP2 is altered (Li and Sudhof, 2004). The lack of BACE1 resulted in significant reduction in the levels of soluble APLP2 (sAPLP2) in the brain, conversely overexpression of BACE1 increased soluble APLP2 levels (Pastorino et al., 2004). In addition, BACE1 deficiency resulted in the inhibition of in C89 C terminal fragment (CTF) in the processing of APP, consistent evidence was observed for APLP2 resulting in a reduction in APLP2 CTF formation (Pastorino et al., 2004). The adverse effect is also true, overexpression of BACE1 increases total sAPLP2 level and an increase in APLP2 CTF levels (Pastorino et al., 2004). Interestingly, BACE1 deficiency did not change intracellular domain (ICD) production amongst the APP family members suggesting that there may be a compensatory mechanism involved for BACE1 activity (Sala Frigerio et al., 2010). Thus, β-secretase cleavage process is believed to be homologous for both APP and APLP2. However, β and γ secretase cleavage of APLP1 and APLP2 does not generate an Aβ peptide due to differences in the sequence (Eggert et al., 2004; Walsh et al., 2007).
1.5.3.2 The α-secretase: the non-amyloidogenic pathway

The non-amyloidogenic pathway involves APP being initially cleaved by α-secretase within the amyloid-β region between the lysine and leucine residues at position 16 and 17 (residues 612 and 613 of APP695) respectively to release the soluble sAPPα fragment (Anderson et al., 1991; Esch et al., 1990; Wang et al., 1991). The remaining membrane bound C terminal of 83 residues termed CTF-α or C83 is then cleaved by γ secretase to generate the p3 peptide and AICD (Haass et al., 1992).

The non-amyloidogenic is the major pathway that occurs in mammalian. The cleavage of APP by α-secretase in mammalians can be undertaken by different members of the A Disintegrin And Metalloprotease (ADAM) family. ADAM 17 was the first identified APP-cleaving enzyme, later ADAM 9 and ADAM 10 were discovered to also cleave APP (Buxbaum et al., 1998; Lammich et al., 1999). Although all have α-secretase activity, it is still not fully established which ADAM member(s) is responsible for the constitutive activity. ADAM 10 is the main α-secretase responsible for the constitutive cleavage of APP in neurons (Kuhn et al., 2010; Vingtdeux and Marambaud, 2012). Neurons that are deficient in ADAM10 showed a reduction in sAPPα. Both ADAM 10 and ADAM 17 can also cleaved APLP2, and similar to APP ADAM10 also constitutively processes APLP2 in neurons (Hogl et al., 2011; Jacobsen et al., 2010). However, the processing of APLP2 is only likely to mediated by ADAM 17 in response to insulin-like Growth Factor-1 (IGF-1) (Jacobsen et al., 2010). Although there are minor differences in α-secretase cleavages, this proteolytic processing pathway is conserved among the APP family members.

1.5.3.3 The γ-secretase

γ-secretase, also known as an aspartyl protease, is a multisubunit transmembrane protease responsible for cleaving the C99 and C83 species in APP produced from β -secretase and α-secretase cleavage respectively (Figure 1.3). The γ-secretase complex comprises at least four subunits presenilin (PS) 1 or 2, nicastrin, anterior pharynx-defective phenotype (Aph-1) and presenilin enhancer 2 (Pen-2) (Kimberly et al., 2003). Evidence for cleavage by γ-secretase of the CTFs in APLP1 is seen in PS1 deficient neurons which resulted in the accumulation of APLP1 CTF (Naruse et al., 1998). In addition, CTFs of APLP1 and APLP2 could be copurified together with the CTF of APP (Gu et al., 2001). Both APLP1 and APLP2 undergo similar γ-secretase cleavage to APP, as shown using γ-secretase inhibitors and genetic deletion of γ-secretase genes (Eggert et al., 2004; Walsh et al., 2003). However, it was observed in primary
neuronal culture studies that the site required for β secretase and γ secretase cleavage for APP is not conserved in either APLP1 or APLP2 (Galvan et al., 2002). Recently, it was evidenced that the direct cleavage of APLP1 is by γ secretase, and it is endo-proteolytically cleaved without previous ectodomain shredding to generate a fragment termed sAPLP1γ (Schauenburg et al., 2018) and is distinct to APLP1. Collectively, these studies suggest the conserved but distinct processing of APP and its family members APLP1 and APLP2.

1.5.4 Functions of APP, APLP1 and APLP2: Evidence from knockout studies
Generating APP-family gene knockout mice has provided a powerful tool to understand the physiological functions of APP and its family members. The APP-family knockout mice studies showed functional differences between the three members in both the nervous system and peripheral tissues (summarised in Table 1.3). Single homozygous deletions of the APP-family gene members (APP-/-, APLP1-/- or APLP2-/-) are viable and fertile with mild phenotypic characteristics. In comparison to both wild-type and APLP1-/- and APLP2-/- mice, APP-/- mice showed a ~10% reduction in overall body weight (Muller et al., 1994; Zheng et al., 1995). APP-/- mice also display an increased agenesis in the corpus callosum (Muller et al., 1996), reduction in locomotor activity and forelimb grip strength (Muller et al., 1996; Muller et al., 1994; Zheng et al., 1995). They also exhibit a decrease in brain weight and size of forebrain commissures, with reactive gliosis in the cortex and hippocampus (Zheng et al., 1996), increased copper levels in the cerebral cortex and liver (Chapman et al., 1999; White et al., 1999). Phenotypically aged APP-/- mice exhibit mild cognitive impairment, behavioural deficits, impairment in long term potentiation (Dawson et al., 1999). At different ages, APP-/- mice also show a reduction in the levels of synaptic marker proteins such as synaptophysin with impairment in neuronal morphology and synaptic function. These results suggest the functional role of APP in synaptic function during the process of ageing (Chapman et al., 1999). Also, hippocampal neurons cultured from APP-/- mice had impaired neurite development and reduced cell viability (Perez et al., 1997). Moreover, co-culturing of APP wild type hippocampal neurons with APP-/- astrocytes resulted in truncated axons and reduced branching compared to co-culturing with wild type astrocytes, indicating APP expression in astrocytes modulates astrocytic mediated neuritic development (Perez et al., 1997). When exposed to Aβ toxicity cortical neurons derived from APP-/- mice appeared to be less susceptible (Lorenzo et al., 2000). In contrast, in another study, cortical and cerebellar neuronal cultures from APP
single knockout mice did not show differences in cell viability even when exposed to Aβ toxicity at various concentrations (White et al., 1998). Collectively, these studies highlight the role of APP in neurite outgrowth, cell adhesion and synaptogenesis.

The minor phenotypic characteristics observed in APP-/- mice indicates that the functional roles of APP can be compensated by its paralogues: APLP1 and/or APLP2. The APLP1-/- mice only show a reduction in body weight (Heber et al., 2000) while APLP2 single knockout mice have no overt phenotype or gross histological abnormalities (von Koch et al., 1997; White et al., 1999). Aged APLP1 knockout mice showed impaired basal transmission and reduced dendritic spines suggesting the importance of APLP1 in synaptically adhesion with the maintenance of basal transmission and dendritic spines density (Schilling et al., 2017). Similar to APP-/- mice, APLP2-/- mice had an increase in copper levels in the cerebral cortex (Needham et al., 2014; White et al., 1999), and both play important but distinct roles in CNS myelination, demyelination and remyelination (Truong et al., 2019).

In contrast to single gene deletions, double-knockout mice APP-/-: APLP2-/- and APLP1-/-:APLP2-/- both exhibit postnatal lethal phenotypes. However, APP-/-: APLP1-/- mice are viable and fertile indicating APLP2 expression alone can ensure viability. Other than the slight decrease in body weight APP-/-:APLP1-/- mice are relatively normal in their phenotype (Zheng et al., 1995). The triple knockout of APP, APLP1 and APLP2 produce mice that die shortly after birth. Importantly, the APP-/-:APLP1-/-:APLP2+/- mouse, i.e. contains only one allele of APLP2, is also lethal (Heber et al., 2000). Analysis of the neuromuscular junction of the lethal APP-family double knockout mice showed defect in neuromuscular junction formation, evident by the decrease in the number of synaptic vesicles, excessive nerve sprouting, widened endplate pattern, reduced apposition of both the presynaptic and postsynaptic proteins and have severely impaired synaptic transmission (Klevanski et al., 2014; Wang et al., 2005; Wang et al., 2009).

These observations strongly suggest that synaptic impairment is a major cause for the lethality phenotype of APP-/-:APLP2-/- and APLP1-/-:APLP2-/- mice. Collectively, these results indicate that APLP2 is an essential member of the APP family and its function as a key survival factor with gene dosage dependent that cannot be compensated for the simultaneous loss of APP or APLP1.
Table 1.3 APP family knockout mouse models and phenotypes.

<table>
<thead>
<tr>
<th>Knockout model</th>
<th>Viability</th>
<th>Cognitive function</th>
<th>Motor function</th>
<th>General phenotype</th>
<th>Biochemical changes</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP-/-</td>
<td>Yes (up to 22 months old)</td>
<td>No apparent abnormalities</td>
<td>↓ locomotor &amp; exploratory activity ↓ grip strength</td>
<td>↓ brain weight ↓ body weight</td>
<td>↓ forebrain commissures size</td>
<td>(Muller et al., 1994; Zheng et al., 1995)</td>
</tr>
<tr>
<td>APLP1-/-</td>
<td>Yes (up to 22 months old)</td>
<td>No apparent abnormalities</td>
<td>No apparent abnormalities</td>
<td>Small deficit body weight</td>
<td>Deficit somatic growth</td>
<td>(Heber et al., 2000)</td>
</tr>
<tr>
<td>APLP2-/-</td>
<td>Yes (up to 22 months)</td>
<td>No apparent abnormalities</td>
<td>↓ locomotor activity</td>
<td>↓ body weight</td>
<td>↑ Cu, Fe (age-dependent)</td>
<td>(von Koch et al., 1997)</td>
</tr>
<tr>
<td>APP-/-: APLP1-/-</td>
<td>Yes (up to 22 months old)</td>
<td>No apparent abnormalities</td>
<td>No apparent abnormalities</td>
<td>↓ body weight</td>
<td>NMJ: no abnormalities</td>
<td>(Heber et al., 2000)</td>
</tr>
<tr>
<td>APP-/-: APLP2-/-</td>
<td>No (perinatal)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>NMJ: ↑ nerve growth, widened endplate, ↓ pre and post-synaptic area and colocalisation, ↓ neurotransmission</td>
<td>(Heber et al., 2000; von Koch et al., 1997; Wang et al., 2005; Wang et al., 2009)</td>
</tr>
<tr>
<td>APLP1-/- : APLP2-/-</td>
<td>No (perinatal)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>NMJ: ↓ pre and post-synaptic area and colocalisation, ↓ neurotransmission</td>
<td>(Heber et al., 2000; Klevanski et al., 2014)</td>
</tr>
<tr>
<td>Genotype</td>
<td>No (perinatal)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>No apparent abnormalities</td>
<td>References</td>
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<td>-----------------------------</td>
</tr>
<tr>
<td>APP-/-; APLP1-/-; APLP2 +/-</td>
<td>No (perinatal)</td>
<td>N/A</td>
<td>No apparent abnormalities</td>
<td>N/A</td>
<td>↓ body weight</td>
<td>(Herms et al., 2004)</td>
</tr>
<tr>
<td>APP-/-; APLP1-/-; APLP2 +/-</td>
<td>Yes (&gt;18 months old)</td>
<td>Not reported</td>
<td>No apparent abnormalities</td>
<td>N/A</td>
<td>No apparent abnormalities</td>
<td>(Needham et al., 2014; Needham et al., 2008)</td>
</tr>
<tr>
<td>APP-/-; APLP2 +/-</td>
<td>Yes (&gt;18 months old)</td>
<td>Not reported</td>
<td>Not reported</td>
<td>↓ body weight</td>
<td>↑ Cu, Fe (age-dependent)</td>
<td>(Needham et al., 2014; Needham et al., 2008)</td>
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1.5.5 The pleiotropic functions of APLP2

Studies of APP family knockout mice have unravelled the functional importance and non-redundant role of APLP2 in survival when compared to its paralogues. Similar to APP, APLP2 has been proposed to possess a number of functions in both the central and peripheral nervous system including synaptogenesis (Wang et al., 2005), neurite outgrowth (Cappai et al., 1999), axonal myelination (Truong et al., 2019), cellular adhesion and signaling (Herms et al., 2004), neuronal differentiation (Bergmans et al., 2010; Shariati et al., 2013), glucose and insulin homeostasis (Needham et al., 2008), brain metal homeostasis (Needham et al., 2014), refractive development (Tkatchenko et al., 2015) and retinal development (Dinet et al., 2016).

In disease conditions, such as myopia, through GWAS, APLP2 was identified to be a susceptible gene (Tkatchenko et al., 2015). Moreover, mice with APLP2 gene deletion showed retinal synaptophathy, a congenital stationary night blindness condition (Dinet et al., 2016). Previous studies have also demonstrated the presence of APLP2 in extracellular matrix and assist in corneal epithelial wound healing (Guo et al., 1998). APLP2 also appears to play a role in cellular growth of several cancer types. APLP2 expression levels have been observed to be upregulated in several human cancers including: pancreatic cancer (Peters et al., 2012; Peters et al., 2015), colon cancer (Moss et al., 2007), breast cancer (Abba et al., 2004), and Ewing’s sarcoma cell lines (Peters et al., 2013a; Peters et al., 2013b). While in other cancer types such as lymphoma cell lines (Tuli et al., 2009b) and in lung neuroendocrine tumours the expression levels of APLP2 is downregulated (Arvidsson et al., 2008). Further studies indicate that APLP2 regulate cancer cell growth and metastasis (Pandey et al., 2015) via associating with major histocompatibility complex (MHC) class I molecules to regulate its cell surface expression and enhancing endocytosis (Peters et al., 2011; Tuli et al., 2009a; Tuli et al., 2008; Tuli et al., 2009b), which will be revisited later in this report.

Collectively, these studies highlight the diversifed functions of APLP2 in both the central and peripheral nervous system. While many functions of APLP2 are comparable to the those ascribed for APP, our knowledge on the physiological and pathophysiological functions of APLP2 remains poorly understood compared to APP.
1.6 APP family and MND: Rationale of the present study

One distinct feature of MND is the significant heterogeneity of the disease both clinically and genetically (Simon et al., 2014). Approximately 20% of MND patients live longer than the usual 4-5 years prognosis, while ~10% of patients can live more than 10 years (Beghi et al., 2011), demonstrating the variability in the age of symptom onset and disease duration. Even in families with a single causative gene mutation, siblings carrying the same mutation can have variation in the age of symptom onset and the rate of disease progression (Penco et al., 2011). One such example is observed in a family that carries the V148G mutation in the SOD1 gene where the disease was diagnosed in a male patient at 25 years with a rapid disease duration of 11 months (Giess et al., 2002). However, his sister who carried the same mutation did not develop the disease at the age of 35 years of age, while his mother was diagnosed with the disease at 54 years of age and died 16 months later. Screening for candidate modifier genes revealed that homozygous mutation in ciliary neurotrophic factor was the most likely cause of variation in this family, which was absent in the sister (Giess et al., 2002). Therefore, it is important to determine which factors, both intrinsic and extrinsic, are responsible for the onset and/or progression of MND.

The molecular mechanisms underlying such differences are not fully understood, however, factors such as genetic modifiers and sex differences have been proposed to contribute to the heterogeneity. Among the pathological changes observed in both MND patients and mutant SOD1 animal models, an increase in protein expression levels of APP was observed (Koistinen et al., 2006; Rabinovich-Toidman et al., 2015; Sasaki and Iwata, 1999). APP levels are upregulated in postmortem spinal cord and muscles tissues from MND patients (Koistinen et al., 2006; Sasaki and Iwata, 1999). Moreover, the increased levels of APP occur in the spinal cord and muscle of SOD1-G93A transgenic mice at symptomatic age (Koistinen et al., 2006; Rabinovich-Toidman et al., 2015). There is a correlation between APP response and muscle fibre atrophy, where APP upregulation was found to occur exclusively in type IIb fibres, which are innervated by fast firing motor neurons, the neurons most susceptible to degeneration in MND.

The role of APP in the central and peripheral nervous system under physiological conditions has been investigated but is still not fully understood (Dawkins and Small,
2014). In disease and injury, APP levels are altered. Following traumatic brain injury (TBI) APP levels are increased and this represents a neuroprotective response (Plummer et al., 2016). APP is upregulated in motor neurons undergoing programmed cell death, ageing neurons, and injured neurons (Itoh et al., 2009; Xie et al., 2000). However, in MND, APP appears to have a detrimental role, where the genetic deletion of APP in SOD1-G93A mice significantly slows down disease progression and promotes motor neuron survival. Assessment of the NMJ innervations in SOD1:APP-/ mice showed reduced NMJ denervation and improved muscle contractility (Bryson et al., 2012). Collectively, these results suggested the important involvement of APP in modulating disease progression in MND.

Since APP can affect MND disease outcomes, it is important and relevant to investigate the role of the other APP-family members as modulators of MND, and better understand the cause and progression of this disease. We chose to investigate the role of APLP2 in MND in vivo due to the essential role APLP2 plays in survival amongst the APP-family members.

In the present study, we first detail the characterisation of APLP2-/ mice by associating motor function with motor neuron and muscle morphology and we compare these observations to APP-/ mice during ageing. We also explored the relationship of APP and APLP2 protein expression during ageing and its expression in both APLP2-/ and APP-/ mice respectively. Next, we characterised the behavioural and biochemical phenotypes of the SOD1-G37R mouse model and examined protein expression levels of APP and APLP2 during disease progression in this transgenic model. Finally, we investigated the role of APLP2 in MND by genetically deleting APLP2 in SOD1-G37R transgenic mice and examined the motor phenotypes in this animal. Since sex differences can be a modifier in MND (reviewed in Section 1.1.2), we also assessed the effects of sex differences by including both female and male groups in the study.
1.7 **Hypothesis and Aims**

This study hypothesizes that the deletion of APLP2 gene will modulate disease progression and phenotype of transgenic SOD1 MND mouse model due to APLP2’s function in neurons.

To address this hypothesis, the following aims were addressed:

1. To examine age dependent effects on neuron and muscle morphology in APLP2-/− and APP-/− mice during ageing (Chapter 3).

2. To examine APP and APLP2 protein expression levels in brain, spinal cord and muscle tissues from APLP2-/− and APP-/− mice during ageing (Chapter 3).

3. To characterise the disease phenotype of SOD1-G37R mice to determine age of disease onset and to assess disease progression (Chapter 4).

4. To examine APP and APLP2 protein expression levels during disease progression in SOD1-G37R mice (Chapter 4).

5. To investigate the effect the genetic deletion of APLP2 has on the disease phenotype of SOD1-G37R mice (Chapter 5).

6. To examine the effect of genetic deletion of APLP2 has on disease mechanisms in SOD1-G37R mice (Chapter 5).
Chapter 2: Material and Methods

2.1 Animals
All mouse experiments in this study complied with the National Health and Medical Research Council code for the care and use of animals for scientific purposes and was approved by the University of Melbourne Animal Ethics Committee (Project Number: 1413304). Mice housing cages contained standard bedding supplemented with tissue paper and cardboard tubes and/or plastic containers for environmental enrichment. Mice were housed on a reverse 12/12-hour light/dark cycle with housing temperature kept at 22 ± 2 °C. Mice grouped 2-5 per cage, sex and age-matched with access to feed and water ad libitum. The experimenter was blinded to age and sex until completion of the behavioural testing. In this thesis, the following abbreviations were used to describe the various mouse genotypes investigated (Table 2.1).

Table 2.1 Abbreviations of mouse genotypes used in the study

<table>
<thead>
<tr>
<th>Full genotype</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>C57BL6</td>
</tr>
<tr>
<td>APP homozygous knockout</td>
<td>APP-/-</td>
</tr>
<tr>
<td>APLP2 homozygous knockout</td>
<td>APLP2-/-</td>
</tr>
<tr>
<td>SOD1-G37R non-transgenic</td>
<td>WT</td>
</tr>
<tr>
<td>SOD1-G37R transgenic</td>
<td>SOD1-G37R</td>
</tr>
<tr>
<td>SOD1-G37R non-transgenic +</td>
<td>WT:APLP2++/+</td>
</tr>
<tr>
<td>APLP2 wild type</td>
<td></td>
</tr>
<tr>
<td>SOD1-G37R non-transgenic +</td>
<td>WT:APLP2-/-</td>
</tr>
<tr>
<td>APLP2 homozygous knockout</td>
<td></td>
</tr>
<tr>
<td>SOD1-G37R transgenic +</td>
<td>SOD1:APLP2++</td>
</tr>
<tr>
<td>APLP2 wild type</td>
<td></td>
</tr>
<tr>
<td>SOD1-G37R transgenic +</td>
<td>SOD1:APLP2-/-</td>
</tr>
<tr>
<td>APLP2 homozygous knockout</td>
<td></td>
</tr>
<tr>
<td>SOD1-G37R transgenic +</td>
<td>SOD1:APLP2+/-</td>
</tr>
<tr>
<td>APLP2 heterozygous knockout</td>
<td></td>
</tr>
</tbody>
</table>
2.1.1 APP-/ and APLP2-/ mice
The APP-/ (Zheng et al., 1995) and APLP2-/ mice (von Koch et al., 1997) are global knockouts and were generated by deleting sequence encompassing the promotor and first exon. Both APP-/ and APLP2-/ mice were backcrossed greater than fourteen times on to a C57BL/6J background resulting in greater than 99.99% C57BL/6J background purity. Genotypes were determined by the polymerase chain reaction (PCR), using the primer sets outlined in Table 2.2.

2.1.2 SOD1-G37R and non-transgenic mice
SOD1-G37R hemizygous mice, line 42, stock #008342 (Wong et al., 1995), were sourced from The Jackson Laboratory (Bar Harbor, USA) on C57BL/6J background. SOD1-G37R and SOD1 non-transgenic line were maintained by backcrossing to the C57BL/6J (sourced from Animal Resources Centre (Western Australia, Australia)) for at least 5 generations before use. Genotypes were determined by PCR using the primer sets outlined in Table 2.2. In the present work the SOD1-G37R mice and the non-transgenic littermates coming from this strain are referred to as SOD1-G37R and WT in Chapter 4 and 5.

2.1.3 Control mice
Control C57BL/6J mice were sourced from the Animal Resources Centre (Western Australia, Australia). All breeding colonies were maintained in the Biomedical School Animal Facility at the University of Melbourne. In the present work the APLP2+/+ littermate control mice generated from the backcrossed of APLP2-/ with C57BL/6J is referred to as C57BL6 in Chapter 3.

2.1.4 SOD1:APLP2-/ mice colony
To establish the SOD1:APLP2-/ mouse line, F1 breeding strategy involved mating the SOD1-G37R and APLP2-/ mice. The SOD1-G37R mice are hemizygous for the SOD1-G37R transgene and when crossed with APLP2-/ mice resulted in WT:APLP2+/- and SOD1:APLP2+/- progeny at a ratio of 1:1.

The F2 breeding strategy involved mating the heterozygous WT:APLP2+/ with SOD1:APLP2+/ mice resulting in six different genotypes 1) SOD1:APLP2+/+, 2) SOD1:APLP2+/+, 3) SOD1:APLP2/-, 4) WT:APLP2+/+, 5) WT:APLP2+/-, 6) WT:APLP2/- at the expected ratios stated in Figure 2.1.
The SOD1:APLP2-/-, SOD1:APLP2+/-, SOD1:APLP2+/+, WT:APLP2-/- and WT:APLP2+/+ (used as the littermate controls) were used for behavioural studies and the WT:APLP2+/- mice were allocated for breeding and colony maintenance (refer to Figure 2.1 for the breeding schematic diagram). Note, only the male SOD1:APLP2+/- and female WT:APLP2+/- mice were used for breeding as the female SOD1:APLP2+/- mice were infertile.

Figure 2.1 Schematic diagram of breeding strategy
In the F1 cross, the hemizygous SOD1-G37R were mated with APLP2-/- mice to generate the heterozygous WT:APLP2+/- and SOD1:APLP2+/- (1:1 ratio). In the F2 cross, the heterozygous WT:APLP2+/- were crossed with SOD1:APLP2+/- to generate 6 different progeny genotypes and the ratio of offspring is also indicated.
2.2 Mouse genotyping

**Genomic DNA extraction:** Tissue was sourced from mouse tail snips or ear clips obtained at weaning age (postnatal day 21) and from mice killed at the end of the study to re-confirm their genotype. Genomic DNA was prepared by lysing the tissue samples in 200 µl of 50 mM of sodium hydroxide (Chem-Supply, Australia) and incubating at 95°C for 10 minutes. The samples were vortexed for 1 minute then neutralised by adding 50 µl of 1M Tris-HCl pH 8.0 (Astral Scientific, Australia). Samples were centrifuged at 12,600 rpm for 5 minutes at room temperature prior to PCR reaction preparation.

**APP and APLP2:** The PCR reaction mix was prepared by combining 1µl of genomic DNA (~50-100ng/µl) (or 1 µl of water as a DNA negative control), 0.3 µl each of both 10 µM forward and reverse primers (APP/APLP2 primers), 0.3 µl of 10 µM pKGNeo for APP and APLP2 (see Table 2.2 for sequences), 6 µl of 2X EmeraldAmp GT PCR master mix (Takara, Clonetech, Australia) and the final volume adjusted to 12 µl with nuclear and DNase free water.

**SOD1:** The PCR reaction mix was prepared by combining 1µl of genomic DNA (~50-100ng/µl) (or 1 µl of water was used as a negative control), 0.3 µl each of both 10 µM forward and reverse primers, 0.3 µl of 10 µM internal forward control and 0.3 µl of 10 µM internal reverse control (see Table 2.2 for sequences), 6 µl of 2X EmeraldAmp GT PCR master mix (Takara, Clonetech, Australia) and the final volume adjusted to 12 µl with nuclear and DNase free water.

**PCR reaction conditions:** PCR was performed using an Eppendorf PCR thermocycler. The same PCR reaction conditions were used for APP, APLP2 and SOD1-G37R genes. The reaction began with an initial denaturing step set 95°C for 1 min, an elongation/annealing step set at 60°C for 1 min, an elongation step at 72°C for 1 min. This was followed by 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. After the final cycle, the reaction mix was cooled to 4°C and kept at this temperature until gel electrophoresis.

**Gel separation of PCR bands:** The PCR products were separated on a 2.5% agarose gel (Bioline, Australia) set in a Tris/Acetate/EDTA buffer (TAE: 40mM TRIS, 20mM acetate and 1mM EDTA (pH 8.0)) containing gelRed stain (Biotium, Gene Target Solutions).
The PCR products were separated at constant voltage of 100 volts for 70 minutes. The gels were imaged using Che-mi-Doc imager (Biorad, Australia).

Table 2.2 List of primers used for genotyping

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Primer nomenclature</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP-/-</td>
<td>Forward</td>
<td>5’-CTG CTG CAG GTG GCT CTG CA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CAG CTC TAT ACA AGC AAA CAA G-3’</td>
</tr>
<tr>
<td></td>
<td>pKGNeo</td>
<td>5’-CCA TTG CTC AGC GGT GCT G-3’</td>
</tr>
<tr>
<td>(von Koch et al., 1997; Zheng et al., 1995)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APLP2</td>
<td>Forward</td>
<td>5’-GCC AAG CTT GAG TCG GTG TAT CCG TGC T-3’</td>
</tr>
<tr>
<td>(von Koch et al., 1997)</td>
<td>Reverse</td>
<td>5’-GCG ACC GGA GGA GAC GCA GAT CGG GAG CTC GCC-3’</td>
</tr>
<tr>
<td></td>
<td>pKGNeo</td>
<td>5’-CCA TTG CTC AGC GGT GCT G-3’</td>
</tr>
<tr>
<td>SOD1-G37R</td>
<td>Forward</td>
<td>5’-GGG AAG CTG TTG TCCCAA G-3’</td>
</tr>
<tr>
<td>(Wong et al., 1995)</td>
<td>Reverse</td>
<td>5’-CAA GGG GAG GTA AAA GAG AGC-3’</td>
</tr>
<tr>
<td></td>
<td>Internal control forward</td>
<td>5’-CAC GTG GGC TCC AGC ATT-3’</td>
</tr>
<tr>
<td></td>
<td>Internal control reverse</td>
<td>5’-TCA CCA GTC ATT TCT GCC TTT G-3’</td>
</tr>
</tbody>
</table>
2.3 Animal monitoring and behavioral analysis

2.3.1 Animal monitoring
All mice were monitored daily using the monitoring and intervention criteria sheets (Appendix B). Mouse body weights were recorded weekly from 3 weeks of age (ie: from weaning age) and the monitoring frequency was increased to three times per week and then daily after 25 weeks of age as the MND symptoms became more progressive and severe (Figure 2.2). Mice were killed when they reached end stage or when SOD1-G37R mice reached their endpoint. The same number of WT:APLP2+/+ and WT:APLP2−/− were killed at the same time for age matching.

![Figure 2.2 Animal monitoring and behavioural testing timeline](image)

*Figure 2.2 Animal monitoring and behavioural testing timeline*

*Progeny generated from F2 cross were monitored daily from birth until weaning age of 3 weeks. Mice were then monitored three times per week and weighed once per week until onset of disease symptoms. Behavioural functions tests using neurological scoring, rotarod assay, stride length test and DigiGait analysis were assessed from 6 weeks of age for SOD1:APLP2−/−, SOD1:APLP2−/+ and SOD1:APLP2+/+, WT:APLP2−/−, WT:APLP2+/+. Mice were killed when they demonstrated at least one severe intervention criteria point.*
2.3.2 Neurological scoring

For each mouse, a neurological score was assessed for both hindlimbs and a rotarod test was done on the same day. Both tests were performed two times per week. The neurological scores were assigned using an amended scale from the neurological scoring system developed by ALSTDI (Leitner et al., 2009; Ludolph et al., 2010). The detailed scoring for hindlimb extension is illustrated in Figure 2.3 and the mouse symptoms outlined in the scoring Table 2.3. Mice body scoring was based on the established body condition scoring method for mice, which is routinely used by the School of Biomedical Sciences Animal Facility at the University of Melbourne for assessing health status in mice (Ullman-Cullere and Foltz, 1999) (Figure 2.3).

Figure 2.3 Hindlimb scoring and mice phenotypes

A) Mice were suspended by the base of the tail and scored based on their leg extension away from the lateral midline. A score of 0 for a full leg extension and showing no motor deficits. The score increases to 1 for partial collapse and 2 for full collapse of the hindlimb when mice showed motor deficits at their symptomatic time points. B) The panel shows mice at symptomatic time point when muscle atrophy, toe clasp and paralysis of the hindlimb are evident. C) The mouse body condition scoring system was amended from (Ullman-Cullere and Foltz, 1999).
## Table 2.3 Mouse neurological scoring

<table>
<thead>
<tr>
<th>Score</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Full extension of hind legs away from lateral midline when mouse is suspended by its tail (suspended 2 - 3 times), and mouse can hold this for two secs (secs).</td>
</tr>
<tr>
<td>0.5</td>
<td>Tremoring of hind legs during tail suspension &amp; partial collapse of leg extensions</td>
</tr>
<tr>
<td>1</td>
<td>Tremoring of hind legs during tail suspension, partial collapse of leg extension towards lateral midline &amp; normal/slightly slower gait</td>
</tr>
<tr>
<td>1.5</td>
<td>Complete/partial collapse of hindlimbs, signs of muscle atrophy hindlimb, forelimb tremoring &amp; sign of toes curl during tail suspension.</td>
</tr>
<tr>
<td>2.0</td>
<td>Complete collapse of hindlimbs, toes curl under at least twice during walking 12 inches, or any part of foot is dragging along cage bottom/table*.</td>
</tr>
<tr>
<td>2.5</td>
<td>Body tremoring and slow walk (male) or wobbly when walking (female), very prominent signs of muscle atrophy in hindlimbs.</td>
</tr>
<tr>
<td>3.0</td>
<td>Rigid paralysis or minimal joint movement, foot not being used for generating forward motion (male) or unsteady when walking, body score &gt;3 (female)</td>
</tr>
<tr>
<td>3.5</td>
<td>Rigid paralysis and no forward motion</td>
</tr>
<tr>
<td>4.0</td>
<td>Paralysis in one or more limbs, body score &gt; 4, mouse cannot right itself within 15 secs after being placed on either side.</td>
</tr>
</tbody>
</table>

*Note: If one hind leg was scored as a 2, food pellets were left on the bedding. If both hind legs were scored as a 2, some food pellets were mashed and left on the bedding and a longer sipper tube was attached to the cage water bottle.*
2.3.3 Rotarod

Rotarod is an instrument used to measure locomotor function in rodents. Two rotarod instruments were used in the study. Both instruments use a rotating rod, which is about 3 cm in diameter, and both have 5 separate experimental chambers. One of the rotarod was covered in a rubber material and it was used for assessing the SOD1-G37R and WT mice (Figure 2.4A) and the data presented in Chapter 4. The second rotarod was made of metal (Figure 2.4B, IITC Life Science, Woodland Hills, CA, USA) and was used to test the SOD1:APLP2 mice genotypes and the data presented in Chapter 5. Both instruments have built in automatic timers for each chamber and the timer begins when the rotarod is started and ends when the mice fall off the rod. In all studies, the mice were habituated to the rotarod instrument six times before the training period began.

Figure 2.4 Rotarod instruments

(A) Rotarod apparatus used to measure the locomotor performance of SOD1-G37R mice and WT non-transgenic mice. (B) Rotarod apparatus used to measure the locomotor performance of SOD1-G37R; APLP2 mice and WT:APLP2 non-transgenic mouse lines.

The habituation trials were conducted on two consecutive days, three times each day. Each trial involved placing a mouse on the rotating rod set at a constant speed of 10 rpm for 300 secs to allow the mouse to explore the apparatus freely. Mice that repeatedly fall off were placed back onto the rotarod until they can stay on the rod for the remainder of the 300 secs. Following habituation, mice were trained on the rotarod for three
consecutive days with the rotation speed initially set to 4 rpm and increasing to 40 rpm over 180 secs. On testing day, mice were transported to the testing room at least 30 minutes prior to testing and weighed before being placed on the rotarod using the same training speed parameters. Mice that were still on the rod after 180 secs were recorded as having no detectable locomotor deficit. Mice that cannot continue on the rotarod for the 180 secs fell on to a padded base. The fall latency was recorded by the experimenter and is defined as the physical fall off the rod or slipping of the hindlimbs (when only the front paws were still grasping on the rod). Rotarod performances were performed and recorded two days per week, with three trials on each day of testing and intervals of 10 minutes of rest between each test trial.

### 2.3.4 Stride length assay
Gait analysis was conducted in a hand built cardboard tunnel apparatus with dimensions of 60cm × 4.5cm × 12cm (length x width x height). The apparatus walkway was lined with a pre-cut strip of white paper. Prior to testing day, mice were trained to run on the instrument three times by placing them at the far end of the tunnel and they were encouraged to move towards the dark box by illuminating the entrance with a desktop lamp. On the test day, the mouse’s hind paws were painted with a water soluble non-toxic black paint and allowed to walk on the sheet of white paper through the cardboard tunnel apparatus leaving paw prints on the white paper (Figure 2.5A). The paw printed strip was allowed to dry, scanned for analysis and record keeping. The progressive change in the distance between the paw prints was used as a measure of locomotor deficiency. For each mouse, 3 measurements were obtained for a) stride length (left and right), b) stance (left and right) and c) sway parameters based on the 4 paw print distances and calculations. The values were then averaged for each parameter and compared between the groups (Figure 2.5B).
Figure 2.5 Stride length assay for gait analysis and representative image of mouse footprints
(A) The cardboard tunnel stride length apparatus including dimensions and study lamp position is shown. (B) Schematic illustration of the gait analysis calculations for stride length (left and right), stance (left and right) and sway parameters measured using 4 paw prints for the left and right side.

2.3.5 DigiGait analysis
Analysis of the mice’s gait was conducted using the DigiGait instrument and software analysis system (Mouse Specifics, Inc, USA) (Figure 2.6A). The DigiGait instrument has long been used for determining gait changes in MND mouse models (Hampton and Amende, 2010). This instrument has a motorized transparent belt upon which the mouse is allowed to walk on as it moves forward at a set speed. A high speed camera located under the belt capture images of the mice’s footfalls during its locomotion and stored on a computer hard drive for subsequent analysis (Figure 2.6B). Prior to testing the mice on this instrument, they undergo a pre-training period for 3 days to acclimatize to the instrument using a range of treadmill speeds to determine the maximum speed the mice could tolerate. After the training period, the mice were tested weekly. For each test, the mouse was placed on the DigiGait and the belt speed set at 10cm/s, 15cm/s or 20cm/s for a period of 15 seconds at each speed. The mice were tested from 7 weeks of age up to the time they are killed.
Figure 2.6 DigiGait Imaging system
(A) DigiGait imaging system apparatus (Mouse Specifics, Inc, USA). (B) Ventral view of a mouse walking on the treadmill and placement of each paw detected from the captured video.

The captured video image data for each mouse was processed using the DigiGait analysis software to calculate a number of gait parameters. The paw placement of each limb was identified through a gait cycle of ~150 frames per second. Each paw placement was graphed as paw area (when the paw was in contact with the treadmill surface) measured over time (in seconds). For each limb, the stride duration was calculated by measuring both the stance duration (when the paw is in contact with the treadmill surface) and swing (when the paw is not in contact with the walking surface). Stance is further subdivided into brake duration, this is the time of decelerating motion and propulsion duration, which is defined as the time for accelerating the motion (Figure 2.7). Each paw placement was viewed by the experimenter and any aberrant strides caused by a mouse jumping, taking partial steps, drags a foot or simply stops moving (Wooley et al., 2005) were excluded from the final analysis.
Figure 2.7 DigiGait analysis output
(A) Representative screen output from the analysis software generated for each of the four limbs of the mouse. (B) Paw area (cm$^2$) measured over time (seconds) were graphed based on the positioning and contact of the paw print of the mouse limbs as it walks on the treadmill. Gait indices from the graphical output were calculated for stride, stance, swing, brake and propulsion.
The DigiGait software automatically generates more than forty different gait parameters for each of the four limbs and are reported in spread-sheet format including; stance, swing, stride, brake, propel and propulsion. A detailed definition of the different parameters generated by the DigiGait analysis software is presented in Table 2.4.

Table 2.4 Definition of the gait parameters used to calculate numerous indices of gait dynamics and posture

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swing Time (second)</td>
<td>The duration when the animal is in swing phase (when paw is not in contact with belt)</td>
</tr>
<tr>
<td>Brake Time (second)</td>
<td>The duration of the braking phase (initial paw contact to maximum paw contact, commencing after the swing phase)</td>
</tr>
<tr>
<td>Propel (second)</td>
<td>The time duration of the propulsion phase (maximum paw contact to just before the swing phase)</td>
</tr>
<tr>
<td>Stance</td>
<td>The time duration of the stance duration (paw contact with belt). Stance duration is equal to the sum of braking duration and propulsion duration.</td>
</tr>
<tr>
<td>Stride Time (second)</td>
<td>The time duration of one complete stride for one paw. Equal to the sum of stance duration and swing duration.</td>
</tr>
<tr>
<td>Stride Length (cm)</td>
<td>The spatial length that a paw traverses through a given stride.</td>
</tr>
<tr>
<td>Stride Frequency (steps/second)</td>
<td>The cadence or the number of times each second that a paw takes a complete stride.</td>
</tr>
<tr>
<td>Paw angle (°)</td>
<td>The angle that the paw makes with the long axis of the direction of motion</td>
</tr>
<tr>
<td>Step angle (°)</td>
<td>The angle made between the left and right hind paw (calculate as a function of stride length and stance width)</td>
</tr>
<tr>
<td>Animal length (cm)</td>
<td>Distance between the beginning of the snout to the base of the tail (viewed ventrally).</td>
</tr>
<tr>
<td>Animal width</td>
<td>Widest part of the body perpendicular to the central axis passing through the body joining the tail base and nose (viewed ventrally).</td>
</tr>
</tbody>
</table>
2.4 Animal end-point

2.4.1 Defining animal end-point
The animal end point is defined as the time in which the mouse was killed. Mice were killed when they met one or more of the following criteria points:

- No longer able to perform a rotarod task.
- The mouse was unable to right itself within 15 seconds of being placed on either side.
- Paralysis was observed in at least one hindlimb.
- Body scoring was < 2.0 (weight loss >15 % of their maximal weight).

2.4.2 Killing and Perfusion
Mice were killed by intraperitoneal injection of a cocktail of xylazine (16 mg/kg body weight) and ketamine (120 mg/kg body weight) prepared in neutral saline solution. The depth of anaesthesia was evaluated by pinching the toes and looking for withdrawal reflex. Once fully unconscious, the animal was pinned on its back and the abdominal cavity opened to expose the sternum. An incision was made on both sides of the diaphragm to open the sternum and to expose the heart in the chest cavity. The aorta was identified and cut and a 23G butterfly needle inserted into the left ventricle of the heart. This needle was connected to a 30 ml syringe filled with ice cold saline solution supplemented with 20U/ml of heparin. Using gentle pressure, cold saline solution was perfused through the mouse for 2 to 3 minutes at a flow rate of 5ml/min. Proper perfusion is indicated by the liver becoming pale. This procedure killed the mouse and the tissues were removed for biochemical and histological analysis (described in 2.4.3).
2.4.3 Dissection and tissue preparation

Tissues were collected for biochemical and histological analysis included the whole brain, spinal cord, and the hindlimb skeletal muscle groups. For biochemical analysis, tissue weights were recorded and the tissues snap frozen in liquid nitrogen and stored at – 80 degrees until processing. For histological analysis, spinal cord tissues were sectioned into four regions to collect separate lumbar spinal cord regions (refer to Figure 2.8 for region of spinal cord used for histology). For all histological analysis the operator was blinded to the genotype groups.

![Figure 2.8 Lumbar spinal cord segments dissection](image)

*Figure 2.8 Lumbar spinal cord segments dissection*

Spinal cord tissue is divided into four regions: cervical, thoracic, lumbar and sacral. Following dissection, spinal cords were cleaned with PBS, and approximately 6mm lumbar section was obtained as indicated in the figure. Lumbar region was processed for histological analysis by immersing in fixatives, while the rest of the spinal cord was frozen in liquid nitrogen for biochemical analysis.

**Spinal cord sections:** The lumbar region was fixed by immersing the tissue in freshly prepared 4% paraformaldehyde and incubating overnight at 4°C and cryo-protected with 30% sucrose for at least 24 hours. Lumbar regions were embedded in Tissue-Tek O.C.T
compound (Sakura FineTek), and snap frozen with liquid nitrogen in isopentane bath. Each mould contains lumbar regions from 5-6 animals of the same phenotype. Lumbar tissues were sectioned serially on a cryostat (Leica) at 20μm thickness. More than six sections were collected for each slide with each section at 200μm apart (refer Figure 2.9A for tissues section arrangement).

![Figure 2.9 Serial cryosectioned spinal tissues slide layout](image)

**Figure 2.9 Serial cryosectioned spinal tissues slide layout**

(A) Frozen spinal cord tissue sections were adhered to PolyLysine coated glass slide. Each slide contained sections from 5 to 6 animals (of the same sex and genotype) and each row of sections corresponded to the same animal group spaced 200 μm apart. Between 7 to 9 rows of serial tissue sections were collected for each animal group. (B) Cartoon illustrating the left and right ventral horn region of a spinal cord section that was selected as the region of interest (ROI) for histological analysis.

**Muscle histology:** The hindlimb skeletal muscle groups gastrocnemius (GA), Tibialis anterior (TA), plantaris (PLA), soleus (SOL), and extensor digitorum longus (EDL) muscles (Figure 2.10) were immersed in Optimal Cutting Temperature (OCT) medium (Tissue-Tek; Sakura Finetek, USA) and laid flat on the end of a syringe and snap frozen with dry ice/liquid nitrogen in isopentane bath. This procedure was used to optimally preserve tissue morphology for subsequent ATPase staining and immunofluorescence. Muscles were stored at -80°C and then warmed to -20 C for sectioning using a cryostat (Leica, Germany). All sections were collected on to poly-lysine coated slides (Thermo Fisher, Australia), air dried at room temperature for at least one hour and stored at -80°C until staining. The GA muscle was sectioned transversely using with 10 μm thick sections cut through the mid-belly region to best observe muscle fibre morphology. The TA
muscle was sectioned longitudinally at 10µm thick and serial sections of the muscle were cut with each section placed 30µm apart on the slide (Figure 2.10B).

![Figure 2.10 Mouse hindlimb skeletal muscle groups](image)

**Figure 2.10 Mouse hindlimb skeletal muscle groups**

(A) Lateral view cartoon representation of mouse hindlimb skeletal muscle groups. (B) Harvested GA muscles sectioned transversely, and TA muscles sectioned longitudinally for histological analysis. Scale bar = 2mm

### 2.5 Histological analysis

#### 2.5.1 Nissl staining of spinal cord sections

Spinal cord frozen sections were air dried to room temperature for one hour and circled with a hydrophobic barrier PAP pen (VWR, Australia). Sections were washed with PBS for 5 minutes to remove OCT compound. Prior to staining the tissues, they would undergo a “defatting step” (to remove the fat content in the tissue and reduce background staining levels) where slides were soaked in 1:1 (v:v) alcohol/chloroform solution overnight at room temperature. Sections were rehydrated by bathing in 100% and 95 % alcohol for 2 minutes each then placed in distilled water. Tissue sections were stained by incubating in 0.1% cresyl violet solution (0.1g cresyl violet acetate, 100 ml distilled water, 0.3 ml glacial acetic acid) for 1 hour in a 37 ºC oven (to improve tissue penetration and enhance evenness in tissue staining). Sections were checked under the microscope for the intensity of the staining and subjected to longer incubations if required. The slides were rinsed in
distilled water for three secs and dehydrated in 70%, 90% and 100% alcohol for 30 secs each. The slides were cleared in xylene solution two times (5 minutes each) and mounted in Safety mounting medium (Trajan, Grale). The spinal cord sections were imaged using a digital slide scanner Panoramic SCAN II (3Dhistech, Hungary) with Carl Zeiss Plan Apochromat 20×/NA 0.8 (Zeiss, Germany). The slides were viewed using Case Viewer software (ver 2.2, 3Dhistech, Hungary).

2.5.1.1 Neuron counting analysis of spinal cord sections
Nissl stained spinal cord sections were imported into Image J/Fiji software (ver. 1.52e, NIH) for semi-quantitative analysis of motor neuron numbers. For each spinal cord section, the left and right ventral horn regions (Figure 2.9B) were selected as regions of interest (ROIs). Neurons were analysed using the manual thresholding command, followed by cell segmentation and particle analysis with neurons having a soma diameter of less than 10 µm excluded from further analysis. A total of seven spinal cord sections was analysed for each animal, and each cut section was at least 200 µm apart.

2.5.2 ATPase staining for muscle tissue
There are two main groups of muscle fibre types, type 1 (slow twitch) and type 2 (fast twitch) fibres, which can be identified by using the adenosine triphosphatase (ATPase) staining procedure. The slow twitch and fast twitch muscle fibre types are classified based on the different myosin heavy chain (MHC) isoforms expressed. The MHC isoforms are responsible for different myosin ATPase activity and speed of contraction (Guth and Samaha, 1969; Salmons and Vrbova, 1969). Pre-incubation with an acid solution (pH 4.3) inhibits the myosin ATPase in type 2 fibre types, causing them to be lightly stained, whereas type 1 fibres are darkly stained at pH 4.3.

The fibre types of the GA muscle from the mouse hindlimb were analysed histochemically by staining for myosin ATPase activity. The frozen transverse muscle sections were allowed to air dry at room temperature for one hour before circling the tissue sample with a PAP pen. Slides were fixed in 4% paraformaldehyde solution for one hour then washed with PBS three times. Two sets of muscle slides were then incubated in Myosin ATPase activity buffer, 1 set at pH 4.3 and the second set at pH 10.2. For Myosin ATPase pH 4.3, slides were pre-incubated in 0.1M acetate buffer at pH 4.3 for 10 minutes. For pH 10.2 analysis, slides were incubated in Baker’s Formal Calcium
solution for one minute. All slides were then washed in distilled water then placed in sodium barbital solution and incubated at 37°C for 30 minutes. Slides were brought to room temperature and washed in distilled water three times, incubated in fresh 1% CaCl₂ for 10 minutes then in 2% CoCl₂ for 10 minutes. Slides were washed thoroughly in distilled water and allowed to develop for 15 secs in freshly prepared 1% ammonium sulfide solution in a fume cupboard. Slides were washed again in distilled water and dehydrated through 70%, 90% and 100% (2 times) alcohol solutions (2 minutes each), cleared in xylene two times (5 minutes each) and mounted in safety mounting medium (Trajan, Grale). Buffer recipes are provided in Appendix A.

2.5.2.1 Muscle fibre typing analysis
Stained muscle sections were imaged using a Zeiss Axioscope 2 light microscope through a 10X objective and images were acquired using Axiocam 503 colour camera with Zenpro software 2011 (Zeiss, Germany). Acquired images were exported in TIF format and imported into Image J/Fiji software (ver. 1.52e, NIH) for further analysis. Total fibre numbers and the composition of each fibre type group were counted manually using the cell counter function. For each muscle fibre type, the cross-sectional area of each muscle fibre cell was measured using ROI function. Over 150 muscle cells were measured per animal.

2.5.3 Immunofluorescence
Cryostat cut spinal cords and the TA muscle sections were warmed to room temperature and allowed to air dry for 1 hour. Using a PAP pen, a circle barrier was drawn around each tissue section/s prior to staining procedures. Muscle tissue sections were fixed in 4% PFA for 1 hour and briefly washed with PBS buffer three times (5 minutes per wash). All tissue sections were permeabilised in permeabilization buffer (Appendix A) for 1 hour, blocked in blocking buffer (Appendix A) for 60 minutes and were incubated with primary antibody diluted in blocking buffer (see Table 2.5 for antibody list) overnight at 4°C in a humidifier chamber. The following day, antibodies were removed, and sections washed with PBS buffer three times (10 minutes per wash) then incubated in fluoro-tagged secondary antibodies (goat anti-mouse or goat anti-rabbit) prepared in blocking buffer containing DAPI dye (2ug/mL) and incubated for 1 hour at room temperature in the dark. For the muscle tissue sections only, neuromuscular junctions (NMJs) were visualised by
adding FITC-alpha-bungarotoxin (CFTM 488A, Biotium, Australia) at 1ug/ml to the secondary antibody buffer solution. All tissues section was washed with PBS three times (10 minutes per wash) and then mounted on to glass slides using antifade mounting medium (Prolong Gold, Invitrogen). The slides were air dried at RT for at least 24 hours in the dark before imaging. The stained tissue sections were visualised through a 20X objective using a Zeiss Axioplan 2 microscope and images taken using a Coolscope snap camera and Zen 2 software (Zeiss, Germany). Captured images were taken using the same exposure settings for all mice genotypes and exported in a TIF format, and the fluorescence intensity levels were quantified using Image J/Fiji software (ver. 1.52e, NIH).

2.5.3.1 Motor neuron and astrocyte analysis
Motor neurons and astrocytes were visualized using CHAT and GFAP antibody (refer to Table 2.5 for antibody dilutions) staining respectively. Quantification in the ventral horn regions of the spinal cord was performed manually using the ROI function tool in ImageJ software program. For neuron counts, the ROI tool was used to trace around cells with positive CHAT staining. For astrocyte counts, the ROI tool was used to trace around cells positive GFAP staining. The integrated intensity mean gray value and area of the region of interest values were exported into an excel spreadsheet file and used to calculate the corrected total cell intensity (CTCI) values. For each spinal cord section, the background intensity was measured in regions known not to contain motor neurons (ie: in the white matter and dorsal horn region).

CTCI values were calculated using the formula below:

\[ \text{CTCI} = \text{Integrated density} - (\text{area selected region x mean gray value of background regions}) \]

2.5.3.2 Neuromuscular junction assessment
The gastrocnemius (Chapter 4) and tibialis muscle (Chapter 5) sections were stained with the FITC-alpha-bungarotoxin to identify the NMJs in the end plates. The innervation status of each NMJ was assessed by determining the level of colocalisation between the muscle fibre end plate (Figure 2.11, α-bungarotoxin, green) and axon terminal of motor neuron (Figure 2.11, synaptophysin, red). Colocalisation level was measured using Imaris software (ver. 9.1, Bitplane, USA) and greater than 150 NMJs were examined in each
animal. For each NMJ, two separate surfaces were created for each channel to allow the creation of a colocalisation surface containing the overlapped region of the two surfaces. The volume of the three surfaces was used to calculate the percentage of colocalization or NMJ innervation.

\[
\text{\% Colocalisation or NMJ innervation} = \frac{\text{Cocalisation surface volume}}{\text{Synaptophysin} + \alpha{-}\text{BTX} \text{ volume}} \times 100
\]

Figure 2.11 Schematic of colocalisation analysis pipeline using Imaris software
Surface rendering of Z stack at the NMJ in tibialis anterior muscle section labelled with (A) \(\alpha\)-bungarotoxin (\(\alpha\)-BTX) and (B) synaptophysin (Syn) in green and red channels respectively. (C) The third surface is created using the “surface-surface coloc” function when two surfaces in red and green are overlapped and (D) the volume of the three surfaces was used to calculate the percentage of colocalization / NMJ innervation

2.5.4 Immunocytochemistry
Tissue sections were warmed to room temperature and allowed to air dry for 1 hour. Using a PAP pen, a circle barrier was drawn around each tissue section prior to staining. All tissue sections were fixed in 4% PFA for 1 hour, briefly washed with PBS buffer three times (5 minutes per wash), permeabilised in permeabilization buffer (Appendix A) for 20 minutes and blocked in blocking buffer (Appendix A) for 60 minutes. Tissue sections were incubated with primary antibody diluted in blocking buffer (see Table 2.5 for
antibody list) overnight at 4°C in a humidifier chamber. The following day, antibodies were removed and tissues washed with PBS three times (10 minutes per wash). Secondary goat anti-mouse or goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) were applied to the sections and incubated for 2 hours at room temperature. Sections were washed with PBS three times (10 minutes per wash) then incubated in diaminobenzidine (DAB) enhancement solution (ImmPACT DAB peroxidase-HRP substrate, SK-4105, Vector labs, Australia). Sections were rinsed with distilled water three times then dehydrated in 70%, 90% and 2 times 100% alcohol once for 2 minutes each. Tissue sections were cleared in xylene two times (5 minutes each) and mounted in Safety mounting medium (Trajan, Gralé) and allowed to dry for at least 24 h before imaging slides. Slides were imaged using a digital slide scanner Pannoramic SCAN II (3Dhistech, Hungary) with Carl Zeiss Plan Apochromat 20×/NA 0.8 (Zeiss, Germany) and the whole sections on the slides were viewed using Case Viewer software (ver 2.2, 3Dhistech, Hungary).

2.5.4.1 Astrocyte and microglia analysis
Quantification of astrocytes and microglia in the spinal cord was performed using antibodies for GFAP and IBA1 markers respectively. The staining area in each image was expressed as a percentage and calculated using ImageJ analysis software (ver. 1.52, NIH, USA). The left and right ventral horn regions of the spinal cord was selected as ROIs. Each ROIs region was then set as an RGB image with automatic thresholding using the “Yen” and “Triangle” settings for astrocytes and microglia respectively. The image was then subjected to “particle analysis” with the exclusion of particles <4µm² to eliminate background noise and the percentage area of field of view (defined as the sum of particle analysis) was determined. For each animal, the percentage area was determined in two regions and the mean values obtained and compared between the groups.

2.5.4.2 Protein expression quantification from DAB stained sections
The left and right ventral horn spinal cord sections were immunoprobed with antibodies specific for GFAP, IBA1, APP and APLP2 protein and visualized by DAB staining immunocytochemistry. Protein expression level was quantified by imaging the DAB stained sections by light microscopy and by measuring the reciprocal DAB intensity levels (Nguyen et al., 2013). However, it is worth noting that there are limitations in quantitative measures of immunohistochemistry for protein expression using DAB due to
the variabilities with the colour intensity of the DAB and the high non-specific binding from IgG secondary antibodies. To overcome these issues, for APP and APLP2 detection, two negative control sections were used, the first includes tissue sections without the antibody of interest and the second includes tissue sections from APP-/− and APLP2-/− mice respectively. Briefly, whole tissue sections were opened in the case viewer software (ver 2.2, 3Dhistech, Hungary) with ventral horn region of the selected, and images saved as TIF format. The TIF format was imported into ImageJ software (ver. 1.52, NIH) with ROIs drawn in the DAB positive region and DAB negative region (for background levels) to measure the area and mean gray values. The relative protein expression in each ROIs was calculated using the following steps of formula:

1. Reciprocal intensity = 250 – mean gray value
2. CTCI = Integrated density – (area selected region x mean gray value of background regions)
3. Intensity (per 100 µm²) = CTCI/area selected region x 100

Where 250 is set as the maximum pixel intensity. The final intensity for each field of view (100 µm²) was then determined by subtracting the CTCI value from the DAB intensity of background tissue sections without primary antibody labelling. The mean CTCI values were obtained in two ventral horn regions of the spinal cord per animal and normalised to the negative controls for comparison between the groups.

2.6 Biochemical Analysis

2.6.1 SDS-PAGE electrophoresis

Brain and spinal cord tissue samples harvested from mice were weighed and lysed at 1:5 w/v in lysis buffer (Appendix A) by passaging several times through a series of 18G and 22G needles followed by an additional homogenization step using a handheld homogenizer while samples were placed on ice. Muscle tissues samples harvested from mice were weighed, then minced with a clean sterile scalpel blade in a petri dish placed on ice and transferred to a centrifuge tube and the muscle lysis buffer added to 1:5 w/v (Appendix A). The tube sonicated with three 30 second bursts. To complete homogenization, all homogenate samples were incubated on ice for 20 minutes then centrifuged at 13,000 rpm for 30 minutes at 4°C. The insoluble material (pellet) was
discarded and the supernatant collected and transferred to a new tube and the protein concentration in the sample quantified by bicinchoninic acid assay (Pierce, Rockford, IL, USA). Lysis buffer was added to the samples to normalize protein levels across the samples to 2 mg/ml.

Proteins were resolved by SDS-PAGE under reducing and denaturing conditions by pre-mixing equivalent amounts of protein samples with 2X or 4X Tris-glycine SDS sample buffer (Appendix A). Samples were heated at 95°C for 15 minutes, allowed to cool for 5 minutes followed by a quick centrifugation step of 10,000 g for 1 minute. Samples were loaded at 20μg into 4-12% NuPAGE Bis-Tris pre-cast gels and the gels were run according to manufacturer’s instructions (Invitrogen, Australia). For both APP and APLP2 detection, positive control samples (C57Bl6 tissues) and negative controls samples (APP-/- and APLP2-/- tissues) were loaded in each blot to ensure APP and APLP2 proteins are detected at the correct size for brains, spinal cord and muscle tissues.

### 2.6.2 Western blot procedure

Resolved gels were transferred onto nitrocellulose membranes (BioRad, Australia) using the wet tank blotting system (BioRad, Australia) in ice cold transfer buffer (Appendix A) set at a constant current of 400 mA for 1 hour duration. Following transfer, the membranes underwent the following incubation steps: the membranes were submerged in the PBS buffer and placed on a rocking platform to prevent membrane from drying. Membranes were then incubated in blocking buffer (Appendix A) for one hour at room temperature and then probed with the primary antibody (see Table 2.5) diluted in PBST (Appendix A) overnight at 4°C. The next day, membranes were washed in PBST buffer three times (5 minutes per wash) then incubated with a secondary antibody conjugated to horseradish peroxidase for two hours at room temperature and finally washed in TBST three times (5 minutes/wash) and once in TBS buffer for 5 minutes (Appendix A). Immunoreactivity was detected by using the enhanced chemiluminescence reagent (ECL-plus, GE Healthcare, UK) and imaged on a ChemiDoc digital imaging system (BioRad, Australia). Protein expression levels were quantitated by densitometry analysis of band intensities using Image J/Fiji software (ver. 1.52e, NIH). The intensity value for each immune-reactive band was normalised to its corresponding housekeeping loading control to account for variability in protein loading across samples.
Table 2.5 Antibodies used for immunohistochemistry and western blot analysis

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Dilution</th>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Immunohistochemistry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Primary Antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APP (22C11)</td>
<td>1:50</td>
<td>Mouse</td>
<td>In house(^1)</td>
</tr>
<tr>
<td>APLP2 (95/11)</td>
<td>1:100</td>
<td>Rabbit</td>
<td>In house</td>
</tr>
<tr>
<td>GFAP</td>
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<td>Mouse</td>
<td>Merck Millipore Australia</td>
</tr>
<tr>
<td>IBA1</td>
<td>1:500</td>
<td>Rabbit</td>
<td>Wako</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>1:500</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>CHAT</td>
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<td>Goat</td>
<td>Invitrogen, Australia</td>
</tr>
<tr>
<td><strong>Secondary Antibodies</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IgG Rabbit (594nm)</td>
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<td>Goat</td>
<td>Invitrogen, Australia</td>
</tr>
<tr>
<td>IgG Mouse (488nm)</td>
<td>1:500</td>
<td>Goat</td>
<td>Invitrogen, Australia</td>
</tr>
<tr>
<td>IgG Goat (488nm)</td>
<td>1:500</td>
<td>Chicken</td>
<td>Invitrogen, Australia</td>
</tr>
<tr>
<td><strong>Specific markers</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>DAPI (461nm)</td>
<td>1:500</td>
<td>--</td>
<td>Sigma</td>
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<tr>
<td>Bungarotoxin (488nm)</td>
<td>1:500</td>
<td>--</td>
<td>Biotium</td>
</tr>
<tr>
<td><strong>Western Blotting</strong></td>
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<td></td>
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</tr>
<tr>
<td><strong>Primary Antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APP (22C11)</td>
<td>1:500</td>
<td>Mouse</td>
<td>In house(^1)</td>
</tr>
<tr>
<td>APLP2 (95/11)</td>
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<td>Rabbit</td>
<td>In house</td>
</tr>
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<td>Rabbit</td>
<td>Abcam, Australia</td>
</tr>
<tr>
<td><strong>Primary Antibodies (Control)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>Cell Signalling, Australia</td>
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<td>Cell Signalling, Australia</td>
</tr>
<tr>
<td>beta-Actin</td>
<td>1:5000</td>
<td>Rabbit</td>
<td>Cell Signalling, Australia</td>
</tr>
<tr>
<td><strong>Secondary Antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG Rabbit (HRP)</td>
<td>1:10,000</td>
<td>Goat</td>
<td>Dako, Australia</td>
</tr>
<tr>
<td>IgG Mouse (HRP)</td>
<td>1:10,000</td>
<td>Goat</td>
<td>Dako, Australia</td>
</tr>
</tbody>
</table>

\(^1\) APP (22C11) antibody was gifted from Dr. Qiao-Xin, Li (The Florey Institute of Neuroscience and Mental Health, Melbourne Australia).
2.7 Statistical analysis

All data are expressed as mean ± SEM with p values of 0.05 or less considered as significant. A student’s *t*-test was used to compare between two groups for both sex and or genotype comparison. Multiple comparisons were assessed using a one-way analysis of variance with Bonferroni’s post-hoc test to compare different genotypes within the same sex groups. A two-way analysis of variances was used when assessing between different genotypes, age and sex, followed by Tukey’s post-hoc tests. To determine the time in which mice showed a reduction in their rotarod performance, a split line regression model using GenStat (ver. 6, VSN international, UK) analysis package were used to fit the performance curve for each animal followed by Student’s *t*-test using GraphPad Prism software (Ver. 7, San Diego, CA, USA) to compare between 2 sex groups. All other statistical analyses were performed using GraphPad Prism software.
Chapter 3: Analysis of motor function, motor neuron formation and muscle fibre composition of APP-/- and APLP2-/- mice during ageing

3.1 Introduction
Ageing is associated with progressive changes in brain structure and function, linking to sensory, motor and cognitive functions as well as changes to body mass composition (Bishop et al., 2010; Keller and Engelhardt, 2013; Simen et al., 2011). Ageing impacts behaviour in both young and aged mice, one of which is motor function decline (Barreto et al., 2010; Shoji et al., 2016). Motor function activities are under the control of the motor unit which is triggered by signals from a motor neuron (Monti et al., 2001). Early motor unit loss in ageing mice is an important driver for physical function deficits observed during ageing (Sheth et al., 2018), and this is associated with denervation and remodelling at the neuromuscular junction (NMJ) (reviewed in Chapter 1 section 1.4) (Arnold et al., 2014; Deschenes et al., 2010; Mantilla and Sieck, 2011).

Both APP and APLP2 are expressed in motor neurons and muscle tissue and its expression at the NMJ is important for NMJ formation and maturation (Akaaboune et al., 2000; Klevanski et al., 2014; Wang et al., 2007; Yang et al., 2005). Despite the important functions of APP and APLP2 at the NMJ, relatively little is known about their role in motor neurons, motor function, and whether there are sex-dependent differences between the male and female APP-/- and APLP2-/- mice.

This Chapter investigated whether the lack of APP or APLP2 expression affected motor neuron formation and motor function during ageing, and whether any changes are associated with sex-related differences. Through analysis of locomotor function and histological assessment, female APLP2-/- and APP-/- mice showed less age-related motor impairments and this was associated with sex-related differences in motor neuron formation and muscle fibre size distribution. Parts of this Chapter have been published in Truong, P.H., Ciccotosto, G.D., and Cappai, R. (2018) Analysis of Motor Function in Amyloid Precursor-Like Protein 2 Knockout Mice: The Effects of Ageing and Sex. Neurochem Res 10.1007/s11064-018-2669-6.
3.2 Results

3.2.1 APLP2 gene deletion causes no change in mouse body weight for either sex

Changes in body weight are a common feature of ageing. Therefore, the body weight of C57BL6 and APLP2-/- mice were recorded at weekly intervals from 5 to 48 weeks of age. The weight of the male mice for both APLP2-/- (p<0.001) and C57BL6 littermate controls (p<0.001) were significantly heavier compared to their female counterparts throughout all ages in this study (Fig. 3.1A, B). The body weight significantly increased as the mice aged from 5 to 48 weeks of age, irrespective of genotype or sex. When comparing the body weight for the different sexes from 5 to 48 weeks of age, no statistical differences were found between the C57BL6 and APLP2-/- cohorts (Figure 3.1C, D).

![Figure 3.1 APLP2 gene deletion causes no change in average body weight for both sexes.](image)

Sex comparison of average body weight in (A) C57BL6 and (B) APLP2-/- mouse groups from 5 weeks of age to 48 weeks of age. Genotype comparison of average body weight of (C) female and (D) male groups. All values represented as mean ± SEM, statistical comparison was performed using a two-way ANOVA and Tukey’s post-hoc test, N=6 for each genotype and sex.
3.2.2 Aged APLP2-/- mice showed abnormal foot clasping phenotype

By visual inspection, from birth until the 48 week time point, the APLP2-/- mice appeared healthy with no observable phenotype regarding their physical or behavioural characteristics compared to the C57BL6 controls.

We examined whether these mice had any motor behaviour abnormalities using the hind-limb reflex test during tail suspension (Figure 3.2). The angle of the hind-limb extension was indistinguishable between C57BL6 and APLP2-/- mice at an early age, but from ~20 weeks of age APLP2-/- mice begin to show abnormal clasping behaviour with retracted hind-limbs and toes as well as a clenched body. In contrast, the C57BL6 control showed normal full hindlimb and toe extensions (Figure 3.2). This neurological abnormality was retained to the 48 week time point (completion of study time point). The clasping response only developed in 30% of APLP2-/- (n=12) mice by 36 weeks of age for both sexes. While the mice displayed a clear clasping abnormality, they did not show any measurable locomotor dysfunction since they displayed normal gait and posture and were tremor free.

3.2.3 Female APLP2-/- mice do not display an age-dependent decline in motor function

To investigate whether the abnormal hindlimb clasping behaviour is associated with motor dysfunction, mice were tested on an accelerating rotarod and the latency to fall was
recorded from 8 weeks of age. The latency to fall for the control mice was significantly decreased at 48 weeks of age compared to the earlier 12 week time point for both the male (p< 0.01) and female (p<0.01) cohorts, but no significant differences were seen when comparing between the sexes (Figure 3.3A). A significant reduction in rotarod performance was observed for both male (p<0.05) and female (p<0.005) APLP2/- mice over the same duration, but unlike the control group the female APLP2/- mice outperformed the male APLP2/- mice on the rotarod from 30 weeks of age. However, this was only significantly different (p<0.05) at the 44 week time point (Figure 3.3B). Female APLP2/- mice began to outperform control females from 30 weeks and were significantly different (p<0.05) from 39 to 45 weeks of age (Figure 3.3C). In contrast, rotarod performance between the male APLP2/- and C57BL6 mice was similar at all ages (Figure 3.3D).

Figure 3.3 Female APLP2/- mice performed better than the female control and male APLP2/- mice on the rotarod test during ageing. Rotarod performance of (A) C57BL6 control and (B) APLP2/- mice beginning at 8 weeks of age. Genotype comparison of rotarod performance of (C) Female and (D) Male mice. Data presented as mean ± SEM. Statistical comparison was performed using a two-way repeated measure ANOVA and Tukey’s post-hoc test, *p< 0.05. N=6 per genotype per sex.
3.2.4 The size distribution of α-motor neurons is altered in APP-/− and APLP2-/− mice

To understand if the reduction in motor performance during ageing is associated with neuronal degeneration Nissl stained neurons in the lumbar spinal cord sections were quantified (Figure 3.4). To consider functional similarities between APP and APLP2, the spinal cord neurons of APP-/− mouse were also assessed to examine if APP expression affected motor neuron morphology.

The α-motor neurons are identifiable in the ventral horn of the spinal cord because they have a large soma with a diameter greater than 20 µm (Friese et al., 2009; Lance-Jone, 1982). The role of α-motor neurons include innervating the extrafusal muscle fibres, which make up the bulk of the skeletal muscle tissue, and they are responsible for generating muscle tension by muscle contraction (Burke et al., 1977; Eccles et al., 1960).

![Nissl stained spinal cord sections of APP-/− and APLP2-/− mice](image)

Figure 3.4 Nissl stained spinal cord sections of APP-/− and APLP2-/− mice. Sections of the ventral horn lumbar spinal cord (L4 to L5) stained with cresyl violet for C57BL6, APP-/− and APLP2-/− mice at 12 and 48 weeks of age (scale bar=100µm).
The number of α-motor neurons present in the ventral horn of the spinal cord was significantly lower in both APP-/- and APLP2-/- mice compared to control mice for both sexes at 12 weeks of age (p<0.0001). Similarly, at 48 weeks of age, APP-/- mice showed a significant reduction in the number of large neurons compared to the age-matched C57BL6 mice (p<0.0001) for both males and females. Interestingly, the 48 weeks old male APLP2-/- mice had significantly less large α-motor neurons compared to male C57BL6 mice (p<0.01) but no statistical differences were found when comparing the female mice at the same age (Figure 3.5A).

We next determined whether APP or APLP2 deficiency influenced the size distribution pattern of motor neurons in the spinal cord. Motor neurons can be identified by the presence of large single nucleolus located within the nucleus and cell soma area >100 μm². It has been previously reported that gamma motor neurons range from 100-250μm², whereas the larger α motor neurons range from 250-1100μm² (Zhou et al., 2013). Therefore, to ensure that we only examine α motor neurons, only neurons with a diameter greater than 10 μm were selected for analysis. The total number of motor neurons between the three male genotypes at 12 weeks of age was not significantly different (Figure 3.5B). In contrast, the female APP-/- mice had significantly fewer (20%) total motor neurons (p<0.01) than female control at 12 weeks. At 48 weeks the total number of motor neurons was significantly reduced in both male and female APP-/- mice (p<0.0001), no difference was observed for APLP2-/- mice in either sex when compared to C57BL6 controls.

To further quantify the changes in motor neuron soma size, we examined the size distribution of ventral horn motor neurons by binning the neuron soma diameter into 3μm increments and expressed it as a percentage of the total number of neurons (Figure 3.5C, D). We observed a significantly higher proportion of the smaller diameter sized neurons (10 to 12 μm) in the APP-/- and APLP2-/- mice for both sexes compared to control mice at 12 weeks of age. While the total number of motor neurons was not different between the genotypes, a higher proportion of smaller neurons were observed in both knockout mice and this may explain the decreased number of the larger diameter neurons observed in the APP-/- and APLP2-/- mice at 12 weeks of age. No difference was observed in the distribution frequency for males at 48 weeks between the three genotypes (Figure 3.5F).
The increase in the smaller diameter neurons persisted in the female APP−/− mice at 48 weeks of age, when compared to both C57BL6 and APLP2−/− mice (Figure 3.5F).

Figure 3.5 Reduction in ventral horn neurons in APP−/− and APLP2−/− mice.

Stereological motor neuron counts in the ventral horn of 7 serial sections of the spinal cord at 200μm apart. (A) Graphs showed the number of neurons with a diameter greater than 20μm per section and (B) the total number of neurons observed in each spinal cord section. Frequency distribution of neurons calibre female and male APP−/−, APLP2−/− and C57BL6 mice at (C, D) 12 weeks and at (E, F) 48 weeks of age respectively. Data represented mean ± SEM, two-way ANOVA with Tukey’s post-hoc test, genotype comparison *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; sex comparison #p<0.05, ###p<0.001, ####p<0.0001; age comparison $p<0.01, $$$p<0.001, $$$$p<0.0001, N=3 per genotype per sex.
3.2.5 Muscle fibre distribution is altered in APP/- and APLP2/- mice

Muscle fibre morphology was assessed histologically to examine whether the changes in the spinal cord neurons of APP/- and APLP2/- mice caused alteration in the muscle fibre caliber.

Skeletal muscle fibres are classified into two types: slow twitch (type I) and fast twitch (type II) muscle fibres. Type I and Type II muscle fibres represent the majority of the muscle fibre types present in the skeletal muscles (Schiaffino and Reggiani, 2011). We stained muscle sections for ATPase activity at pH 4.3 because it can discriminate the type I (dark brown colour) from the type II (beige light brown colour) fibre types.

Histological analysis of the cross-sectional gastrocnemius (GA) muscle at 12 weeks showed no gross morphological differences in the pattern of fibre distribution between all three mice groups (Figure 3.6A, B). This was confirmed following quantitation of type I and II fibres distribution (Figure 3.6C, D). At 48 weeks, female APLP2/- mice had a significantly higher percentage of type I fibres compared to age-matched APP/- and C57BL6 mice (p<0.001, Figure 3.6C). The proportion of type I fibres in female APLP2/- at 48 weeks was also higher compared to the age-matched male APLP2/- mice (p<0.001) and 12 week old female APLP2/- mice (p<0.0001, Figure 3.6C).

When we compared the cross-sectional area (CSA) of both type I and II muscle fibres across the groups and found a significantly lower CSA in type I fibres for APP/- mice compared to age-matched C57BL6 and APLP2/- (p<0.05) for both sexes. The CSA of type I fibres were comparable between the control and the knockouts for both sexes at 48 weeks (Figure 3.6E). Comparing the 12 versus 48 week groups indicated a significant increase in CSA for type I fibres in male C57BL6 control (p<0.01) but no change for female C57BL6 control mice (Figure 3.6F). In contrast, APP/- mice showed a significant increase in CSA for both sexes (p<0.05) as they age, while no differences were observed for APLP2/- mice.

The CSA for type II fibres were significantly lower for both male APP/- and APLP2/- mice at 12 and 48 weeks compared to C57BL6 control mice (p<0.05). Although a trend for a reduction was observed for female APP/- mice at 12 weeks, this was not statistically significant. In contrast, APLP2/- mice showed a significantly higher CSA in type II
fibres compared to C57BL6 control mice at the same age group (p<0.01). At 48 weeks female APP-/- mice also demonstrated a reduction in CSA of type II fibres compared to female control mice (p<0.05), but no differences were found for female APLP2-/- mice. An age comparison showed CSA was significantly increased for all the male genotypes at 48 weeks only. When comparing between sexes, CSA for type II fibre was significantly lower for female WT (p<0.05) and higher for APLP2-/- mice (p<0.001), compared to male mice at 48 weeks (Figure 3.6 E, F). No sex differences were observed for the different APP-/- groups. Collectively, these results identified age and sex differences in muscle fibre proportion and size, and this may explain the changes seen in the number of spinal cord neurons, their size and distribution patterns in the APP-/- and APLP2-/- mice.

Figure 3.6 Muscle analysis of gastrocnemius muscle from 12 and 48 weeks old APP-/- and APLP2-/- mice. (A) Representative myosin ATPase stain (pH 4.3) of cross-sections from GA muscle of female and male C57BL6, APP-/- and APLP2-/- at 12 and 48 weeks (scale bar=100µm). Calculated (B, C) percentage distribution and (D, E) fibre CSA determined for type I and type II fibre types respectively. Values are mean ± SEM, two-way ANOVA with Tukey’s post-hoc test, genotype comparison *p<0.05, **p<0.01, ***p<0.001,
Skeletal muscle composition is correlated with muscle mass and is critical for muscle function during ageing (Narici and Maffulli, 2010). We investigated whether the morphological changes in the muscle fibre in APP-/− and APLP2-/− at 12 weeks is associated with a change in muscle mass. This was done by measuring the tissue wet weight of different hindlimb muscles and correcting them for mouse body weight. The results showed the ratio of muscle mass and body weight was not statistically different between the three genotypes for either soleus (SOL), plantaris (PLA) and extensor digitorum longus (EDL) muscles (Figure 3.7). While there was a trend for a reduction in muscle wet weight/mouse body weight ratio for tibialis anterior (TA) muscle in APLP2-/− mice, this was not statistically different. Similarly, there was a trend in the reduction for GA muscle/mouse body weight for both female and male APP-/− and APLP2-/− mice groups, but this was not statistically different to C57BL6 control mice (Figure 3.7). Therefore, altered motor neuron size distribution (Figure 3.5) and muscle fibre distribution (Figure 3.6) observed in the APP-/− and APLP2-/− mice cannot be explained by hindlimb muscle atrophy in these mice.

**Figure 3.7** No change in relative muscle wet weight in APP-/− or APLP2-/− mice. The ratio of muscle wet weight (mg) relative to mice body weight (g) of C57BL6, APP-/− and APLP2-/− mice at 12 weeks of age for (A) male and (B) female groups All values represented as mean ± SEM, one-way ANOVA followed by Bonferroni’s post-hoc test, N=3-4 for each genotype and sex.
3.2.6  APLP2 protein expression levels are increased in the brain and spinal cord of APP-/ mice

To determine whether APP gene expression modulates APLP2 expression, central and peripheral tissues from male and female APP-/ mice were examined at two time points. As expected, APLP2 protein expression was absent in brain, spinal cord and muscle lysates from APLP2-/ mice (Figure 3.8A-C). APLP2 levels were significantly higher in the brain lysates of 12 weeks old male APP-/ mice compared to control mice (p<0.001) and no differences were found between the respective female cohorts (Figure 3.8D). Comparison between sexes showed higher levels of APLP2 protein expression in the male APP-/ mice (p<0.001, Fig.3.8D). However, in the brains of 48 weeks old APP-/ mice, APLP2 protein expression levels were similar to age-matched C57BL6 controls for both sexes (Figure 3.8D). These results contrasted with the spinal cord data since APLP2 levels were significantly higher (p<0.05) in the 12 weeks old APP-/ female compared to male C57BL6 controls, but no difference was observed in the age-matched male APP-/ mice. When we compared APLP2 protein expression across the two age groups we found a significant reduction (p<0.05) in APLP2 protein levels in the brains of 48 week old male APP-/ mice. We also found a significant reduction in APLP2 protein expression levels in the spinal cord of 48 week old male APP-/ mice (p<0.05, Figure 3.8E). In contrast to brain and spinal cord tissues, a significant reduction in APLP2 protein level was observed in the hindlimb muscle of male APP-/ mice at 12 weeks of age but no statistically significant difference was observed at 48 weeks of age for both sexes (Figure 3.8F). These results highlight the compensatory expression of APP and APLP2 is tissue specific and is influenced by sex and age-dependent changes.
3.2.7 APP protein expression levels are increased in the brain and spinal cord of APLP2-/− mice

Next, we determined whether APLP2 gene expression modulates APP expression in brain, spinal cord and muscle during ageing. No detectable APP immunoreactivity was present when immunoblotting in APP-/− mice tissue lysates (Figure 3.9 A, B). In APLP2-
/- mice, APP levels were significantly higher in the 12 week old brain lysates for both female (p<0.05) and male (p<0.0001) mice when compared to control mice. Comparing APP levels between females and males, a significant increase was seen for male APLP2/-/- mice at 12 weeks (p<0.0001). However, this level was significantly reduced in the male APLP2/-/- at 48 weeks of age (p<0.0001), and the levels were comparable to control mice for both sexes. APP expression in the 48 week old mice was significantly lower compared to 12 week old mice for both female (p<0.05) and male (p<0.0001) respectively (Figure 3.9D). Unlike the brain, APP protein expression levels in the spinal cord of APLP2/-/- mice were similar across the 12 and 48 weeks of age for both sexes when compared to C57BL6 control mice (Figure 3.9E).

In contrast to the presence of a distinct single 100 kDa band for APP in neuronal tissue extracts (Figure 3.9 A, B), we observed three distinct immunoreactive bands for APP in hindlimb skeletal muscle extracts with apparent molecular weights of ~100kDa, ~55kDa and ~25kDa in both sexes and ages for all three mouse genotypes (Figure 3.9C). APP immunoreactivity in APP/-/- mice skeletal muscle is consistent with reports from our lab (White et al., 1998) and others (Slunt et al., 1994) reporting the 22C11 antibody cross-reacting with APLP2. Therefore, the 22C11 bands in APP/-/- muscle may be APLP2 and not APP protein. A reduction in APP protein levels (100kDa) was detected in APLP2/-/- muscle at 12 weeks, for both sexes, but was only significantly different for females (p<0.01). Analysis of the 55kDa fragment yielded a similar result. For all bands, APP levels were similar between APLP2/-/- and C57BL6 mice at 48 weeks of age (Figure 3.9 F, G, H). In contrast, the 55kDa and 25kDa 22C11 fragments were significantly higher (p<0.001) in the older female control group but comparable for the male group. These results suggest a differential expression pattern for APP fragments in the hindlimb muscles for both C57BL6 control and APLP2/-/- mice during ageing. Taken together, these results highlight a similarity to APP/-/- mice where we observed tissue and sex-specific expression pattern for APP in APLP2/-/- mice.
Figure 3.9 APP protein levels are upregulated in the brain and spinal cord of APLP2-/- mice at 12 weeks.

Representative western blot images depicting the protein expression of levels of APP (~100kDa) and GAPDH (loading control) for (A) brain, (B) spinal cord and (C) hindlimb muscle of APP-/- mice at 12 and 48 weeks of age. Densitometric quantification of APP protein levels are expressed relative to GAPDH for (D) brain, (E) spinal cord and (F-H) muscle normalised to 12 weeks old C57BL6 control mice. Data represented mean ± SEM, two-way ANOVA and Tukey’s post-hoc test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n=5-7 per genotype and sex.
3.2.8 Evaluation of BACE1 and NRG1 protein expression levels in different tissues of APP-/- and APLP2-/- mice

β-Secretase 1 (BACE1) is a type I transmembrane aspartyl protease and can cleave APP and APLP2 (Hogl et al., 2011; Vassar et al., 1999) and Neuregulin I (NRG1) (Hu et al., 2006; Luo et al., 2011). Both BACE1 and NRG1 are important in axonal myelination and synaptic function (Hu et al., 2016; Hu et al., 2006; Laird et al., 2005; Luo et al., 2011; Ryu et al., 2012; Willem et al., 2004). NRG1 signalling is involved in the non-amyloidogenic processing of APP in neuronal cells (Kim et al., 2018b). Therefore, to determine whether APP or APLP2 affects BACE1 and NRG1 protein expression, their levels were analysed in APP-/- and APLP2-/- mice.

We found BACE1 (p<0.0001) and NRG1 (p<0.05) protein levels were significantly elevated in the brains of 12 week male APP-/- mice compared to age-matched C57BL6, APLP2-/-, and female APP-/- mice (Figure 3.10A). No differences were seen in the APP-/- mouse group at 48 weeks. BACE1 and NRG1 protein levels were comparable between WT and APLP2-/- mice in both age groups for both sexes. Comparing BACE1 and NRG1 protein levels at 12 and 48 weeks, we detected significantly higher levels of BACE1 and NRG1 in the male APP-/- mouse group at 12 weeks (p<0.05). However, only BACE1 protein expression was significantly higher (p<0.01) in APLP2-/- 12 week males. In contrast, BACE1 and NRG1 protein expression levels were not significantly different in the spinal cord and muscle tissues of APP-/- and APLP2-/- for both age groups (Figure 3.10 B, C).
Figure 3.10 No change in BACE-1 and NRG-1 protein expression levels of APP-/– and APLP2-/– mice at 12 and 48 weeks old.

Representative immunoblot images (i) and densitometric analysis in the (A) brain, (B) spinal cord and (C) hindlimb muscles of C57BL6, APP-/– and APLP2-/– mice at 12 weeks and 48 weeks of age for (ii) BACE-1 (~62kDa) (iii) and NRG-1 (~45-55kDa) protein levels. Relative protein levels were normalised to loading controls and C57BL6 at 12 weeks. Data represented mean ± SEM, two-way ANOVA and Tukey’s post-hoc test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, N=5-7 per genotype and sex.
3.3 Discussion
This Chapter investigated the role of APP and APLP2 in motor neuron and muscle function during ageing by examining motor activity and muscle composition in the young (12 week old) and older (48 week old) APP and APLP2 single knockout mice. The results demonstrated both APP and APLP2 have distinct functional roles in motor neuron and muscle function. Moreover, APP/- mice also showed reduced motor neuron numbers in both age groups and was associated with altered motor neuron and muscle fibre size distribution. In contrast, APLP2/- mice showed sex-dependent alterations in motor neuron and muscle fibre size distribution during ageing. Comparable body weights were observed between APLP2/- and C57BL6 controls, as they age, and these results were similar to the originally reported description for APLP2/- mice (von Koch et al., 1997).

The original publication of the APLP2/- mouse model reported no overt behavioural and motor phenotypes (von Koch et al., 1997). However, when we examined the hindlimb clasping in our APLP2/- cohort at ~5-6 months of age, we observed a clear behavioural phenotype. Interestingly, the mice that exhibited the hindlimb clasping did not show locomotion impairment in the rotarod test. Abnormal hindlimb responses such as exhibiting a paw clasp response without displaying signs of a locomotor deficit or displaying motor coordination deficit (rotarod, coat hanger and stationary beam) has been described for the transgenic mice overexpressing the βAPP gene with the Swedish mutation (Lalonde et al., 2005; Lalonde et al., 2002; Lalonde et al., 2003). It accumulated Aβ in the brain but did not cause a hindlimb and paw clumping response in these APP transgenic mice due to the SJL genes in the genetic background for the mouse model (Lalonde et al., 2004). There are no reports about the commonly used C57BL/6J mouse strain regarding hindlimb or paw clasping response and how this alters with ageing (Lalonde et al., 2002; Lalonde and Strazielle, 2011). We also observed no clasping response in the C57BL6 control used in this study, suggesting that the clasping phenotype observed in the APLP2/- mouse model is due to the loss of APLP2 gene expression rather than the genetic background. These results show APLP2 is important in neurological function and that the loss of APLP2 expression can cause the hindlimb clasping responses.
Normal ageing is associated with progressive changes in the brain, and impairment of both sensory and motor function (Bishop et al., 2010; Keller and Engelhardt, 2013; Simen et al., 2011). Ageing studies on muscle changes have been mainly performed in male C57BL/6 mice (Graber et al., 2015; van Norren et al., 2015) with only a few studies looking at sex differences (Ashworth et al., 2015; Fearing et al., 2016; Tucker et al., 2016). Therefore, we analysed male and female mice separately to determine if there are sex-specific differences.

In the longitudinal study of rotarod performance during ageing, we observed an age-dependent decline in motor function in both sexes of C57BL6 control mice. Our results agree with previous reports of reduced motor function and rotarod performance in 18 month old C57BL/6 mice (Barreto et al., 2010). Aged C57BL/6 mice showed reduced neuromuscular strength at 8 to 12 months of age, but no difference in motor performance in the single timepoint rotarod test (Shoji et al., 2016). Previous reports show aged mice have impaired sensorimotor dependent behaviours and their physical activity also decreased with age (Benice et al., 2006; Dean et al., 1981; Fahlstrom et al., 2011; Shoji et al., 2016). Similar to previous studies (Ashworth et al., 2015; Kovacs and Pearce, 2013; Tucker et al., 2016) we also found no differences in the rotarod performance between female and male control mice on the C57BL/6 background. Interestingly, while male APLP2-/- mice showed a similar reduction in rotarod performance compared to the littermate controls, we observed a significant improvement in the motor performance (less of a decline) in the females APLP2-/- cohort.

Sexual dimorphism has been observed to influence the age of onset, disease progression and disease severity in many common human diseases including cardiovascular disease, autoimmune disease and asthma (Ober et al., 2008). Despite this clear sexual dimorphism, female rodents are not often used in behavioural studies due to the potential variability in results that may be caused by the female oestrous cycle. However, this has been demonstrated in C57BL/6J mice that locomotion activity and reflex responses in female mice remain stable across all phases of the oestrous cycle (Meziane et al., 2007). Recent reports also demonstrated that the oestrous cycle plays a minor role in mouse physical performance (Aguiar et al., 2018), therefore including both sexes in animal studies is important. Female mice exhibit better motor and behavioural performance outcomes in
C57BL/6 mice (Tucker et al., 2016) and rat (O’Connor et al., 2003) following TBI. The basis for these differences are still unknown, but in female rats, the effect of the oestrous cycle on motor performance post TBI was believed to be a minor contributing factor (Wagner et al., 2004) and it had no effect on motor and cognitive tasks (Monaco et al., 2013). Female mice, which exhibited better performance in the rotarod test compared to male mice, was reported for the AD triple transgenic mice (3xTg-AD, expressing mutant human APP, presenilin and tau) and was presumably due to the lower body weight in female mice and associated lower latency to fall while on the rotarod test (Oore et al., 2013). These results suggest that the oestrous cycle may play a subtle role in the physical performance of mice. However, it doesn’t rule out the possibility that the deletion of APLP2 may cause alterations in sex hormones leading to female mice with better levels of motor activity during ageing.

At the cellular level, we tested whether there would be a correlation between the reduction in motor neurons and muscle morphology and found significant differences in the spinal motor neurons and muscle fibre composition between young and old APP−/− and APLP2−/− mice. There are three main classes of motor neurons in the motor nucleus; α-motor neuron, gamma-motor neuron and beta-motor neuron (Manuel and Zytnicki, 2011) (reviewed in Chapter 1 section 1.4.1.1). The α-neuron were identified by their large soma size (cell body diameter > 20 µm) and they are responsible for the majority of skeletal muscle contraction activity (Friese et al., 2009; Lance-Jone, 1982). There are conflicting reports regarding the loss of motor neurons in mice during ageing (Chai et al., 2011; Chung et al., 2017; Valdez et al., 2010). A reduction in α-motor neurons were found in aged human and animals and hypertrophy observed in the remaining α-neurons (Jacob, 1998; Tomlinson and Irving, 1977), other’s reported no difference in the number and size of motor neurons in aged mice compared to adult mice (Chai et al., 2011; Maxwell et al., 2018).

In our study, we observed a significant increase in α-motor neurons number with no change in soma size and a decline in motor function in the C57BL6 control mice for both sexes at 48 weeks of age when compared to the respective sex cohort at 12 weeks. It is possible that the increase in body mass during ageing maybe correlated with the increase motor neurons numbers (Watson et al., 2012). In addition, a recent study looking at α-
motor neurons in primates and mice which demonstrated that although motor function deteriorates with ageing the soma of α-motor neurons and innervating synaptic inputs were retained in the aged spinal cord of both species (Maxwell et al., 2018). Further studies regarding the size and type of these motor neuron pools with the particular muscle groups that they innervate are required.

On the other hand, APP/-/- mice at both 12 and 48 weeks of age showed a reduction of α-motor neurons and muscle fibre CSA compared to age-matched C57BL6 control mice. This is in alignment with the reduced body weight, locomotor activity and forelimb grip strength observed in APP/-/- mice (Muller et al., 1994; Senechal et al., 2008; Tremml et al., 1998; Tremml et al., 2002; Zheng et al., 1995). The reduction in APP/-/- mice body weight may explain the decline in CSA for both young and old age groups. While APP/-/- mice at 3 months showed reduced locomotor activity but not at 8 months of age (Senechal et al., 2008), 12 month old APP/-/- mice showed reduced spine density in the cortex and the hippocampus with impairments in long-term potentiation and spatial learning (Dawson et al., 1999; Senechal et al., 2008; Tyan et al., 2012). This indicates the age-related decline of locomotor activity was not augmented in APP/-/- mice and it may have been compensated with ageing.

These results may explain the improvement in type II muscle CSA in APP/-/- at 48 weeks compared to 12 weeks of age. We too observed a significant reduction in α-motor neurons in APLP2/-/- mice at 12 weeks of age, however, the reduction in α-motor neurons was only observed in male APLP2/-/- and not in the 48 week female group. The reduction in α motor neuron number in the male group at 48 weeks is also in agreement with the reduction in CSA of muscle fibre type II for male APLP2/-/- at 48 weeks of age but unchanged for the female group. Thus, these results suggested that the impaired synaptic connection between motor neuron and muscle fibre groups in the C57BL6 and male APLP2/-/- mice groups may contribute to the reduced motor function during ageing while unchanged for female APLP2/-/- mice.

Taken together, the combined data indicate that the APP/-/- mouse display either a selective loss of the larger motor neuron or they have a developmental alteration leading to an increase in the number of smaller motor neurons. These data also indicate there are sex differences in the distribution of neurons in APLP2/-/- mice as they age. The sex-
related differences in motor neuron number and muscle fibre distribution pattern may explain why female APLP2-/- have superior motor function performance compared to male APLP2-/- mice and C57BL6 control mice during ageing.

The reduction in muscle mass and the reduction of motor neurons in ageing has been suggested to be associated with NMJ remodelling, an effect which precedes muscle fibre atrophy and could be involved with initiating muscle loss (Arnold et al., 2014; Deschenes et al., 2010; Mantilla and Sieck, 2011). Denervation at the NMJ is a major contributor to the diminished motor function observed in older mice (Chai et al., 2011; Chung et al., 2017; Hepple and Rice, 2016; Li et al., 2011; Valdez et al., 2010), while impairment at the NMJ follows degeneration in the muscle fibres in the neck muscles of aged mice (Li et al., 2011) and significant loss of NMJ innervation during ageing in mice can be rescued by caloric restriction and exercise without motor neuron loss (Valdez et al., 2010). While the NMJ innervation status of the APP-/- and APLP2-/- mice in this study was not performed, it is known that both APP and APLP2 single knockout mice do not display obvious alterations in the NMJ structure. As opposed to APP/APLP2 double knockouts with significant impairment in the neuromuscular synapses, which were shown to attribute compensatory roles of other APP-family members (Heber et al., 2000; Klevanski et al., 2014; Muller et al., 1994; von Koch et al., 1997; Wang et al., 2007; Yang et al., 2005; Zheng et al., 1995) (reviewed in section 1.5.4). Given the roles of APP and APLP2 at the NMJ, it may explain the significant alterations seen in motor neuron distribution and muscle fibre size in APP-/- and APLP2-/- mice during ageing. Collectively, these data suggest APP and APLP2 affect muscle development and/or function.

The APLP2 molecule shares significant structural and functional similarity with APP, therefore, APLP2 could compensate for the loss of APP expression in APP-/- mice (von Koch et al., 1997; Zheng et al., 1995). To examine possible compensatory roles of APP and APLP2 during ageing, we performed western blotting analysis of CNS (brain and spinal cord) and PNS (muscle) tissues of C57BL6, APP-/- and APLP2-/- mice. We observed a significant increase in APLP2 protein expression levels in the brain and spinal cord of male and female APP-/- mice respectively at 12 weeks of age. These results contrast to previous mRNA studies in APP-/- mice, which showed mRNA levels of both APLP1 and APLP2 were unchanged in the brains of APP-/- mice (Zheng et al., 1995).
Similarly, APP/- mice cortical neurons also showed no difference in APLP1 and APLP2 protein levels (White et al., 1998). However, sex differences in protein expression of APLP2 and APP in these studies were not examined. Sex differences in BACE1 and NRG1 protein expression were also observed in APP/- mice with significant elevation of BACE1 and NRG1 expression found only in the brain of male APP/- mice at 12 weeks.

In the developing muscle fibres, APP can be detected as early as embryonic day 16 (E-16) and found to localise to the NMJ at postnatal day 0 (P0) (Akaaboune et al., 2000). APP protein expression is mainly restricted to the synapse and colocalised with acetylcholine receptors (AChRs) at P5, and P15 (Akaaboune et al., 2000). In our study, APP protein expression was also detected at high levels in skeletal hindlimb muscle. However, we detected no compensatory expression of APLP2 and APP in either APP/- or APLP2/- mice. On the contrary, both male and female APP/- mice at 12 weeks of age showed a significant reduction in APLP2 protein expression level. This result provides further support towards functional roles for APP and APLP2 at the NMJ.

Apart from the reduced locomotor activity, we described earlier (Figure 3.3), APP/- mice at P0 was reported to show impaired colocalization between presynaptic motor neurons proteins and the postsynaptic skeletal muscle endplate AChR (Wang et al., 2005). Electrophysiological studies also demonstrated that the conduction properties of APP/- mice showed a reduction in the compound action potentials in the spinal cord (Li et al., 2016). In summary, these results identify redundant roles of both APP and APLP2 in NMJ formation and maintenance of muscle function. Further studies are needed to understand the relationships between muscle fibre size, motor unit connectivity and morphological changes that occur during ageing, and the effect of factors such as sex and exercise.

3.4 Conclusion
In this current Chapter we highlight the relationships between muscle fibre size, motor unit connectivity and morphological changes during ageing and the importance of sex. We found that the deletion of APLP2 gene in female mice can reduce age-related motor function impairments. We also observed age and sex differences in motor neuron and muscle fibre size distribution for both APP/- and APLP2/- mice. These alterations can be explained by compensatory activities between the APP gene family members.
Chapter 4: Characterisation of the disease phenotype and APP/APLP2 expression in SOD1-G37R mice during disease progression

4.1 Introduction

The clinical features of sporadic MND patients and those with familial MND are generally indistinguishable. Among the 140 different SOD1 gene mutations described for familial MND, the G37R mutation is found in exon 2 of the SOD1 gene (Rosen et al., 1993). Early studies suggested that the mechanism of disease underlying MND is the loss of activity, however subsequent in vitro studies of G37R quickly demonstrated that while some mutants had reduced activity the mutant G37R enzyme retains its full specific dismutase activity both in vitro and MND patient, suggesting a gain of toxic function (Borchelt et al., 1994). Several SOD1 mutations have been reported to correlate with a more or less severe course of the disease (Cudkowicz et al., 1997). For instance, patients with G37R mutation were reported to show earlier age of disease onset, longer survival, and a high level of penetrance, while a shorter survival is correlated with patients exhibiting the A4V mutation (Cudkowicz et al., 1997; Garcia-Redondo et al., 2002). Neuropathological examination of a patient with G37R mutation showed neuronal degeneration of the spinal cord and brainstem with the presence of Lewy like inclusions (Inoue et al., 2002).

The SOD1-G37R MND mouse model used in the study are hemizygous for the 12kb human genomic DNA encoding the G37R mutation (glycine to arginine substitution at codon 37) and is expressed under its endogenous promoter (Wong et al., 1995). Compared to the endogenous expression of SOD1 in the normal mouse, these transgenic mice express 4 to 12-fold higher SOD1 levels and have a corresponding 5 to 14 fold increase in SOD1 activity (Cleveland et al., 1996; Wong et al., 1995). Among the four G37R founder lines that develop associated neuropathology of MND, mouse line 29 and line 42 are the most widely used and they differ mainly in transgene copy number (Cleveland et al., 1996; Wong et al., 1995). The SOD1-G37R line 42 has higher transgene copy number in comparison to SOD1-G37R line 29, and the disease phenotype emerges at a much
earlier time point for line 42 compared to line 29 (reviewed in Chapter 1, Section 1.2.1). The pathological features seen in this transgenic mouse line reflect those seen in familial MND human cases with the G37R mutation including neurofilament inclusions, selective motor neuron degeneration of the brain stem and spinal cord (Wong et al., 1995). In the SOD1-G37R line 42, which expresses more SOD1, the neuronal vacuolar pathology was observed to precede axonal degeneration, muscle atrophy and motor neuron death (Wong et al., 1995). In comparison to the other SOD1 mouse models of MND, transgenic mice with the G37R mutation develop the disease for a longer duration, hence a longer survival compared to the more popular G93A mouse model (Gurney et al., 1994; Wong et al., 1995). Therefore, the SOD1-G37R (42) is ideal for the current study, allowing for more flexibility in the experimental design.

Despite the benefits of utilising in vivo transgenic mouse models in delineating the underlying causes of MND pathogenesis, therapeutics potential from research using these SOD1 mouse models have failed to translate into respective clinical applicability (Benatar, 2007; Petrov et al., 2017). The main reasons for these therapeutic failures are most likely due to the selection and validation of the mouse models used during the preclinical stages. Therefore, it is of utmost importance to select, validate and characterise the in vivo animal model used in the study to ensure that the chosen animal model best replicates those seen in human disease. In this Chapter, we extended the work of Wong and colleagues in 1995 (Wong et al., 1995) who characterised the SOD1-G37R (line 42) transgenic mouse model and evaluated whether the disease phenotypes in the SOD1-G37R mice was sex-dependent. This analysis will establish the foundation for the subsequent studies (Chapter 5) analysing the effect of APLP2 expression on the disease phenotype of SOD1-G37R. This Chapter will also examine the expression profile of APP and APLP2 during disease progression and determine whether this is sex-dependent.
4.2 Results
To generate the SOD1-G37R transgenic and non-transgenic (WT) littermate controls, hemizygous SOD1-G37R mice (line 42, stock #008342) on C57BL/6J background purchased from the Jackson Laboratory were crossed with C57BL/6J mice in our animal facility (see Chapter 2: Material and Methods). All offspring of the SOD1-G37R breeding mice were genotyped for the SOD1 gene by PCR using genomic DNA isolated from tail clip samples (Appendix C, Figure C. 1). Western blot analysis of spinal cord tissues of the SOD1-G37R transgenic mice confirmed the presence of human SOD1 protein expression in SOD1-G37R transgenic mice and not in the WT littermate controls (Appendix C, Figure C. 1).
4.2.1 No sex differences in the reduction of mice body weight in SOD1-G37R mice

Mice were weighed weekly starting 7 weeks of age until they reached end stage (approx. 27 to 30 weeks). Male mice were significantly heavier than their female counterparts for both SOD1-G37R and WT mice throughout the period of testing (p<0.05). Before the onset of symptoms developing (~17 weeks), SOD1-G37R mice have similar body weight to the WT controls for both sexes. From 17 week of age time point, both female and male SOD1-G37R mice begin to lose body weight relative to the weight gain seen in the WT mice resulting in a divergence in animal weights during this latter period (Figure 4.1A). In addition, the body weight of SOD1-G37R mice for both male and female groups decreased comparably as disease symptoms developed and peaked at around 18 weeks of age as shown by the percentage of body weight change relative to the maximal weight curve (Figure 4.1B).

Figure 4.1 Changes in average body weight of G37R expressing transgenic mice

(A) Weekly average body weight in male and female SOD1-G37R and WT littermate control mice. (B) Calculated percent weight change of male and female SOD1-G37R mice relative to maximal weight. Data are mean ± SEM, repeated measures two-way ANOVA, followed by Bonferroni post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, N=3-5.
4.2.2 No sex differences in the survival of SOD1-G37R mice

To determine survivability, end-stage was determined when mice displayed either a 15% loss in their body weight compared to their maximum body weight, an inability to right themselves within 15 secs after being placed on either side or when they exhibited a neurological score of 4. Although female SOD1-G37R mice showed a slight delay in their mean survival time compared to their male counterparts (male 26.6 ± 0.7 weeks, female 27.7 ± 0.4 weeks), this was not statistically significant (Figure 4.2).

Figure 4.2 No change in survival between female and male SOD1-G37R mice.

*Kaplan-Meier survival curve for females and males SOD1-G37R mice and in WT littermate controls.*
4.2.3 Rotarod test indicate disease onset is delayed in female SOD1-G37R mice

Motor coordination and motor learning were assessed twice weekly using an accelerating rotarod, starting from 7 weeks of age. Prior to recording, the mice were trained on the rotarod for 5 consecutive days to allow them to get acclimatised to the instrument. Male SOD1-G37R mice demonstrated a learning curve in their motor function, where they showed a marked improvement in their motor performance during the first 2-3 weeks of training. Their rotarod performance gradually reached a plateau at ~10 weeks of age. As disease symptoms developed both sexes of SOD1-G37R mice began to display a decrease in the rate of their latency to fall from the rotarod. Compared to male SOD1-G37R mice which had an early decreased in the latency to fall at ~22 weeks, the female SOD1-G37R mice had a delay in rotarod dysfunction, and this was significant with a decreased in latency to fall at 24-27 weeks (p<0.05, depicted in grey region Figure 4.3). As expected, rotarod performance was maintained throughout the testing period for WT littermate control mice and they showed no significant decreased in the latency to fall (Figure 4.3).

![Figure 4.3 Female SOD1-G37R showed a delay in disease onset on the rotarod test.](image)

**Figure 4.3 Female SOD1-G37R showed a delay in disease onset on the rotarod test.**

*Locomotor function was assessed using the rotarod assay and recorded as latency to fall for female and male SOD1-G37R mice and their WT littermate control mice. Grey region indicated the time points at which significant differences were detected between male and female SOD1-G37R. Data are mean ± SEM, two-way ANOVA followed by Tukey’s post-hoc test, *p<0.05, N=3-5.*
4.2.4 Neurological scores showed no sex-dependent differences in the SOD1-G37R mice

The neurological deficit of mice was assessed using the neurological scoring system adapted from the ALS Therapy Development Institute (ALSTDI) (Leitner et al., 2009; Ludolph et al., 2010). Mice were scored on both the left and right hindlimbs from 8 weeks of age using a modified neurological scoring system described by ALSTDI (refer to Chapter 2, Table 2.3). All mouse groups from 8 to 13 weeks of age had a neurological score of 0 (Figure 4.4). The neurological score for male and female WT mice remained at 0 throughout the study. The neurological scores for both sexes of the SOD1-G37R mice were significantly increased in the right hindlimb from 16 weeks of age (p<0.05) and continued to increase until 30 weeks of age compared to WT littermates (Figure 4.4). There was no significant difference in the score for the onset of symptoms between male (14.55 ± 0.25 weeks) and female SOD1G37R (15.05 ± 0.36 weeks) mice in either hindlimb (Figure 4.4).

![Figure 4.4 Neurological score of male and female SOD1G37R mice from 8 weeks of age](image)

Assessment of Neurological scores for (A) left and (B) right hindlimbs of SOD1-G37R male (blue and female (red) and WT littermate male (black) and female (grey) mice. SOD1-G37R mice exhibited increases in neurological scores from 16-17 weeks of age for both hindlimbs. Data present as mean ± SEM, repeated measures two-way ANOVA, followed by Tukey’s post-hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
4.2.5 Gait abnormalities are delayed in female SOD1-G37R mice

Motor function was also investigated using the stride length assay. This assay measures gait parameters for stride length, stance and sway for left and right hindlimbs (refer to Chapter 2, Section 2.3.4). The analysis revealed that gait parameters were similar for either sex in SOD1-G37R mice from 8 to 23 weeks of age compared to the WT littermates (Figure 4.5), despite exhibiting a detectable hindlimbs extension impairment using the neurological scoring system at ~14 - 15 weeks of age (Figure 4.4). Beginning 24 weeks of age we observed a clear separation in the stride length for both male and female SOD1-G37R mice when compared to the WT littermates. We observed a significant separation in the stride length for male and female SOD1-G37R mice from 24 and 26 weeks of age respectively compared to WT littermate controls (Figure 4.5A, B). Like the stride length changes, similar differences were observed for stance (Figure 4.5D, E). While for sway changes, a significant reduction was observed only at 28 weeks of age in the SOD1-G37R mice compared to WT control mice for both sexes (p<0.05, Figure 4.5G, H). Compared to their female counterparts, male SOD1-G37R mice exhibited an earlier decrease in the relative stride length and stance parameter, and this was significant at 27 weeks of age (p<0.01, Figure 4.5C, F). In summary, gait abnormalities detected in the SOD1-G37R mice overlaps with the motor coordination results from the rotarod assay, overall suggesting that male mice displayed a slightly earlier disease onset compared to their female counterparts.
Figure 4.5 Gait analysis of SOD1-G37R mice hindlimbs

Motor function measured using footprint analysis with graphs showing the relative (A-C) stride length, (D-E) stance and (G-I) sway for both left and right hindlimbs comparing between female, male, and between sexes respectively for WT and SOD1-G37R mice groups. Values represented mean ± SEM, two-way ANOVA with Tukey’s post-hoc test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, N=3-5.
4.2.6 Female SOD1-G37R displayed a delay in motor function deficit compared to male mice

The above data showed the earliest disease symptoms were detected using the neurological score function assay which detected symptoms at around 14 weeks of age for both female and male SOD1-G37R mice. More severe symptoms developed following a decline in maximal body weight which occurred at ~17 weeks of age for both sexes. Using the stride length assay, gait deficit is detected earlier in the male mice at ~21 weeks compared to female mice at ~23 weeks of age, and this was significant at end stage of disease at 27 weeks of age (p<0.05). This is followed by a progressive decline in motor performance in the rotarod assay, where male mice also displayed a slightly earlier disease onset. Collectively these results showed that in our SOD1-G37R colony, female mice demonstrated a slight delay in disease onset compared to male mice, and this is detected towards the later stages of disease when symptoms are more prominent (Figure 4.6). The slight delay in symptoms onset for female SOD1-G37R agrees with the moderate sex specific differences seen in the human cases of MND, where men are slightly more affected than women (Manjaly et al., 2010).

![Figure 4.6 Behavioral profile for disease onset of SOD1-G37R mice](image)

**Figure 4.6 Behavioral profile for disease onset of SOD1-G37R mice**

*Schematic representation to model disease onset of SOD1-G37R mice, with symptoms severity increases with age. Disease onset was defined by the age that shows the first sign in an increase in neurological scores, a decline from maximal body weight, decrease in gait deficit of stride length assay and a decrease in rotarod performance in comparison to the respective male and female WT littermates. Female mice showed a slight delay in disease onset compared to male mice.*
4.2.7 Decreased gastrocnemius muscle mass in the SOD1-G37R mice at end stage is attributed to the atrophy of muscle fibre and defective synapses at the NMJ

To test the validity of using the SOD1-G37R mouse model we examined if it recapitulates the neuropathological features present at the different stages of the human MND disease. Different hindlimb skeletal muscles were harvested from SOD1-G37R mice at the presymptomatic (12 weeks) and end stage timepoints and compared to age-matched WT mice (Figure 4.7A). Quantification of muscle wet weight/body weight of the different skeletal muscle of the hindlimbs revealed significant reduction in the GA muscle weight at end stage in the SOD1-G37R mice (p<0.05, Figure 4.7B). When comparing sexes at disease end stage, both male and female SOD1G37R mice showed a significant reduction in the weights of GA muscles compared to their WT age-matched littermate controls (p<0.05). There was no significant difference between male and female SOD1-G37R mice (Figure 4.7C).
Figure 4.7 Disease end stage SOD1-G37R mice showed decreased gastrocnemius muscle mass.

(A) Images of hindlimb skeletal muscle harvested from SOD1-G37R and their WT littermate controls at the end stage of the disease. (B) The calculated ratio of muscle wet weight (mg) relative to body weight (g) was determined in male mice at pre-symptomatic (12 week) and disease end stage. (C) Comparison between male and female mice cohorts at disease end stage. Data represented mean ± SEM, one –way ANOVA with Bonferroni’s post-hoc test, *p<0.05, ns: not significant, N=3-5.
Since the GA muscle is altered in the SOD1-G37R mouse, histological assessment of the muscle fibre subtypes was determined. Histological assessment of GA cross sections revealed that the physiological pattern of type 1 and type 2 muscle fibre morphology was disrupted in end stage SOD1-G37R mice and they exhibited substantial atrophy of type 2 fast twitch muscle fibres, as reflected by the shrunken appearance of the myofiber groups (indicated by the black arrow in Figure 4.8).

![ATPase pH 4.3](image)

Figure 4.8 Histological examination of gastrocnemius muscle of SOD1-G37R mice revealed muscle fibre atrophy
Representative images of GA muscle cross sections stained with ATPase (pH 4.3) from (A) aged-match WT littermate control and (B) SOD1-G37R mice at end-stage of disease (~30 weeks of age). Atrophy of the fast twitch muscle fibre groups (black arrow) was observed in the SOD1-G37R mouse.
The NMJ is the site of communication between motor nerve axons and skeletal muscle fibres (reviewed in Chapter 1.4) (Li et al., 2018). To determine if muscle atrophy observed in the SOD1-G37R mouse is associated with the defective synapses at the NMJ, the structural integrity of the NMJ was examined. Motor nerve terminals and endplate acetylcholine receptor (AChR) clusters in NMJs were stained using synaptophysin and alpha-bungarotoxin (BTX). At the NMJs the AChR clusters are intact and the motor nerve terminals innervated with AChR clusters to form synapses for signal transmission. In the end stage SOD1-G37R mice, denervation of the NMJs was observed, as evidenced by the collapse of the NMJ structures, with a decrease in synaptophysin staining as well as a 41% overall reduction in the number of innervated NMJs (p<0.001, Figure 4.9).
Figure 4.9 Significant denervation at NMJ in SOD1-G37R mice
(A) Immunofluorescence micrographs of the NMJ from SOD1-G37R mice at end stage of disease and age-matched WT controls. To identify the motor end plates, longitudinal cryo-sections of the gastrocnemius muscle were immunolabelled for presynaptic markers, anti-synaptophysin (red) and α-bungarotoxin (green, AChR) co-stained with DAPI (blue, nucleus). (B) The number of innervated NMJ as assessed by colocalization of synaptophysin and α-bungarotoxin staining. Data present as mean ± SEM, student-
t-test. ***p<0.001, N=6-8. Scale bar = 5μm.
4.2.8 Immunohistological analysis reveals significant motor neuron degeneration and astroglisis in the spinal cord of SOD1-G37R mice at disease end stage

To determine if the motor dysfunction in the SOD1-G37R mice is associated with motor neuron death and gliosis, as seen in human MND, we stained the spinal cord of SOD1-G37R mice at disease end stage for Choline Acetyltransferase (CHAT) and GFAP. Immunohistochemistry of SOD1-G37R spinal cord tissue showed significant motor neuron death (decreased CHAT) and increased astroglisis (GFAP) (Figure 4.10).

These results confirmed previous reports that at disease end-stage the SOD1-G37R mice exhibit neuropathological features including muscle atrophy, neuronal loss and astroglisis (Wong et al., 1995).

Figure 4.10 Decreased CHAT staining intensity and upregulation of GFAP levels in the spinal cord of SOD1G37R mice.

(A) Representative fluorescence micrographs of the lumbar ventral horn spinal cord sections from end stage SOD1-G37R mice and age-matched WT control mice immune-stained for Anti-Choline Acetyltransferase Antibody (CHAT) and anti-GFAP. (B) Quantification of mean intensity levels for CHAT and GFAP positive cells from the spinal cord sections. All data were normalised to WT age-matched littermate controls. Data presented as mean ± SEM, student-t test, **p<0.01, ***p<0.001, N=3-5. Scale bar = 50μm.
4.2.9 Protein levels of APP and APLP2 are increased in the spinal cord of SOD1-G37R mice

The protein expression levels of APP and APLP2 was measured in lysates from brain stem, cerebellum, spinal cord and muscle harvested from SOD1-G37R and age-matched WT littermate mice at three time points during disease progression: 12 weeks (presymptomatic), 22 weeks (symptomatic) and ~28 weeks (end stage) (Figure 4.11).

Figure 4.11 SOD1G37R mice disease progression timeline

*Schematic representation of disease timeline of SOD1G37R mice. For protein analysis SOD1-G37R mice and age-matched WT littermates at 12 weeks (pre-symptomatic), 22 weeks (symptomatic) and ~28 weeks (end stage) old mice were killed and tissues harvested.*
Full length APP and APLP2 protein bands were detected by western blot analysis. In both cerebellum and brainstem the protein levels of APP and APLP2 did not significantly differ between SOD1-G37R and WT littermates (Figure 4.12).

Figure 4.12 No change in APP and APLP2 levels in the brain of SOD1-G37R mice during disease progression

*Immunoblot images and densitometric quantification of (A) brain stem (B) and cerebellum lysates for (ii) APP and (iii) APLP2 protein expression levels in the WT and SOD1-G37R mice at 12 weeks (pre-symptomatic), 22 weeks (symptomatic) and 28 weeks (end stage) old mice. Data are normalised to loading control protein (GAPDH) and expressed as a ratio of the WT presymptomatic sample for respective tissue samples. Data present as mean ± SEM, *p<0.05, two-way ANOVA with Bonferroni’s post-hoc test, N=4-6.*
In spinal cord tissue, APP and APLP2 levels were significantly elevated in the SOD1-G37R mice at disease end stage (p<0.05) when compared with age-matched WT controls (Figure 4.13A). Expression of APP and APLP2 was almost 3-fold higher in the SOD1-G37R mice at end-stage compared to the presymptomatic and symptomatic stages (p<0.01, Figure 4.13A).

In the hindlimb muscle, APP levels in the SOD1-G37R mice did not change at any disease stage. Although we observed a similar trend of decreasing APP levels (Figure 4.13B) in the WT mice, this was not significant. While in the WT mice, APLP2 expression levels were significantly reduced at ~28 weeks compared to the 12 week old mice, but was not different to the SOD1-G37R mice at any disease stage (Figure 4.13B).
Figure 4.13 APP and APLP2 protein expression is increased in the spinal cord during disease progression of SOD1G37R mice.

Immunoblot images and densitometric quantification of (A) spinal cord (B) and hindlimb muscle lysates for ii) APP and iii) APLP2 protein levels in the WT and SOD1-G37R mice at 12 weeks (pre-symptomatic), 22 weeks (symptomatic) and 28 weeks (end stage) old mice. Data are normalised to a loading control and expressed as a ratio of the WT presymptomatic sample for respective tissue samples. Data presented as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA with Bonferroni’s post-hoc test, N=4-6.
4.2.10 APP and APLP2 protein levels are increased in the spinal cord of SOD1-G37R mice in a sex-dependent manner

Since APP and APLP2 protein levels are increased in the spinal cord of SOD1-G37R at disease end stage, we evaluated if this is sex-dependent (Figure 4.14). While we saw upregulation of APP levels in the spinal cord of SOD1-G37R mice for both sexes, APP levels are significantly lower in the female compared to male SOD1-G37R mice at disease end stage (Figure 4.14B). We also found the levels of APLP2 were elevated in both male and female SOD1-G37R mice groups, however, it was only significant in male mice (Figure 4.14C). These results indicate that the significant elevation of both APP and APLP2 in the spinal cord of SOD1-G37R mice is sex-dependent.

Figure 4.14 APP and APLP2 protein expression is increased in the spinal cord during disease progression of SOD1G37R mice.

(A) Immunoblot images and densitometric quantification of (B) APP and (C) APLP2 protein levels in the spinal cord of male and female of age-matched WT controls and SOD1-G37R mice at disease end stage. Data are normalised to GAPDH levels and WT male group and compared to the respective WT female and male groups. Data presented as mean ± SEM, *p<0.05, ****p<0.0001, one-way ANOVA with Bonferroni’s post-hoc test, N=5-10.
4.3 Discussion

Mouse models are a powerful tool to help understand MND and how gene mutations in SOD1 mediate their deleterious effects of specific subsets of neurons. Many SOD1 based mouse models exist, however, the positive therapeutic outcomes observed in these animals have thus far failed to translate into beneficial effects in human clinical trials (Benatar, 2007). This raises the need for a better characterisation of these animal models and standard outcome measures that can be applied to achieve reproducible, reliable, consistent and constructive data (Ludolph et al., 2010). This Chapter aimed to provide a detailed analysis of disease onset and progression of motor deficits, and examine if these are sex-dependent, in the SOD1-G37R mouse model. The time course of disease progression in the SOD1-G37R mice was monitored by motor function, body weight loss, rotarod performance, survival and a modified neurological scoring system over the 6-7 months life span.

Weight loss is a simple and unbiased parameter to characterise disease progression in the SOD1-G37R mice and maybe associated with motor neuron degeneration over the course of the disease (Feeney et al., 2001; Nau et al., 1995). In our study, we observed a decrease in body weight in the SOD1-G37R mice. Our studies are consistent with previous reports of weight loss in the SOD1-G37R line that both male and female SOD1-G37R mice consistently weighed significantly less than WT control mice from disease onset (Hilton et al., 2018; Kang and Rivest, 2007). Body weights for both sexes of SOD1-G37R (line 42) and WT control mice were similar until approximately 17-18 weeks of age then a noticeable decline in body weights began to occur in these affected mice until disease end stage. This is consistent with previous studies which reported an increase in body weight in the SOD1G37R mice with sexes combined from 5 weeks until 18-19 weeks of age where the body weights start to decline as disease symptoms become apparent (Hilton et al., 2018). Various hypotheses have been proposed to explain weight loss in MND patients including muscle fasciculation, the increase of respiratory efforts, hypermetabolism and reduced food intake caused by depression (Bouteloup et al., 2009; Holm et al., 2013; Vaisman et al., 2009). However, body weight alone is a non-specific characterisation of the disease time course as it can be affected by other conditions such as dehydration, malnutrition, genetic background (Alexander et al., 2004).
Different locomotor function techniques have been used to evaluate disease onset and progression in SOD1 transgenic mouse models (Filali et al., 2011; Olivan et al., 2015). Of these, rotarod is one of the most utilised assays to assess locomotor function and detect early motor impairment (Olivan et al., 2015). Rotarod performance in the SOD1-G37R mice was not significantly different from control mice until disease symptoms developed. Using rotarod performance, “motor symptom onset” was defined as the point of decline in the performance from their plateau performance. When we compared this with their neurological scores, visible signs such as limb tremor and hind-limb splay defects were detectable. The point of rotarod performance decline was observed at around 25-26 weeks for female SOD1-G37R mice and 23-24 weeks of age for male mice. Our results are consistent with other studies for the SOD1-G37R mice with rotarod performance beginning to decline at ~21 weeks (McAllum et al., 2013) and ~23 weeks (McAllum et al., 2015), and was significantly decreased during disease progression (Hilton et al., 2018). In contrast, studies in low transgene copy number SOD1-G37R (line 29) mice detected the deficits at a much later time point of 32 weeks of age (Filali et al., 2011). However, rotarod measures of SOD1-G37R mice in these studies did not compare between sexes. Nevertheless, the results from our studies support these studies and indicate that SOD1-G37R mice displayed a progressive decline in locomotor function during disease progression.

In MND patient disease symptoms are believed to start focally and then progress to other areas of the motor system over time (Walhout et al., 2018). Therefore, evidence for the differential onset of disease progression between the left and right side is interesting and informative as the onset mechanism could be different from the pathology underlying disease progression in MND. To detect differences in limb performance we measured the neurological performance of the mice separately for the left and right hindlimb from 7 weeks of age. We found that right hindlimbs show an earlier disease phenotype compared to the left hindlimb. The neurological scores showed that the first clinical sign of disease in the SOD1-G37R mice occurred from 14 weeks of age first in the right hindlimb, and the symptoms were delayed by 1 week in the female compared to male mice. This is indicated by the increase in neurological scores for limb tremor, hindlimb splayed defect
and body scoring. Similarly, differences in the left and right limb onset have also been reported in human MND. In a study of MND patients with upper limb onset the frequency of upper limb onset in the right limb is higher compared to those of the left limb (Ravits et al., 2007). This difference between right and left sides could explain the earlier disease symptoms observed in the right limb of the SOD1-G37R mice. This may also be related to handedness, where studies have reported that for patients with upper limb onset, there was a significant concordance between the side of limb onset and handedness (Turner et al., 2011).

Similar to the rotarod test, we also found a marked difference in the gait pattern between the WT and the SOD1-G37R mice. Our results are in partial agreement with a previous publication of SOD1-G93A transgenic mice, which reported a significant impairment in stride length and sway at symptomatic stage of the disease (Alves et al., 2011; Filali et al., 2011). Differences in stride length between female and male SOD1-G37R mice have not been reported. However, previous studies on SOD1-G93A have demonstrated an early gait deficit in the female compared to the male groups (Alves et al., 2011). In contrast, our results showed that the female SOD1-G37R mice exhibited a delay in disease symptoms onset with a significant reduction in the stride length at 26 weeks compared to the male groups at 23 weeks of age. This suggests that the delay in the gait abnormalities in the female SOD1-G37R mice results in better motor performance on the rotarod compared to the male SOD1-G37R mice.

To determine the validity of the SOD1-G37R mouse model in recapitulating the neuropathological features in the human MND, histological analysis was performed in the hindlimb muscles and spinal cord. We observed a significant decrease in GA muscle wet weight at disease end stage for SOD1-G37R mice and this was associated with fast twitch/type II muscle fibre atrophy. Our result is supported by electrophysiology studies of SOD1 mice, in which quantification of isometric forces showed an earlier decrease in motor units in fast twitch muscle compared to slow twitch soleus muscle following disease symptoms onset (Hegedus et al., 2007, 2009). The differential vulnerability between muscle groups can be explained by the mitochondrial oxygen consumption, which differs between different muscle types, where slow twitch muscles have a lower consumption to those of glycolytic fast-twitch muscles (Leclerc et al., 2001). Consistent
with previous reports that showed degeneration of distal axons, branching and alterations in NMJ structure (Clark et al., 2016), we also saw a large decrease in the innervated junctions in the GA muscles of SOD1-G37R mice. The detachment of the NMJ, and the selective vulnerability in muscle fibre groups to atrophy can be explained by motor neuron pools that innervate these muscle fibres. Within the spinal cord, these motor neuron pools lie within the ventral horn, where we observed significant motor neuron degeneration and increased inflammatory response. Our results support previous reports that fast twitch fast fatigable motor neurons degenerate before the slow motor neurons in MND mouse models (Frey et al., 2000; Hegedus et al., 2008; Pun et al., 2006). Together these results suggest that the motor dysfunction presented in these mice is associated with motor neuron degeneration, synaptic dysfunction and muscular atrophy.

Protein levels of APP and APLP2 in the brain stem, cerebellum, spinal cord and hindlimb skeletal muscles of the SOD1-G37R and non-transgenic littermate mice were compared at pre-symptomatic, symptomatic and end-stage of disease. The results confirmed previous observations of increased APP expression in the spinal cord and muscle of MND patients, and during disease progression in transgenic SOD1 mice (Koistinen et al., 2006; Rabinovich-Toidman et al., 2015; Sasaki and Iwata, 1999). Our results also demonstrated a substantial increase in APP protein levels in the spinal cord of mutant SOD1-G37R mice, however, this was only observed in mice at disease end stage. Similar to APP, APLP2 levels was significantly upregulated in the spinal cord of SOD1-G37R mice at disease end-stage. APLP2 is processed by the same proteolytic enzymes as APP, and both proteins are expressed at the synaptic vesicles and presynaptic active zone, thus they may be activated in a similar fashion sharing similar functions (Fanutza et al., 2015; Heber et al., 2000; Herms et al., 2004; von Koch et al., 1997).

The investigation into sex-dependent effects showed higher levels of both APP and APLP2 in male SOD1-G37R mice compared to female mice. Although studies have reported an increase in APP protein levels in the muscle of MND patients (Koistinen et al., 2006), we did not see significant changes in APP and APLP2 levels in skeletal muscle of SOD1-G37R mice as disease progressed. This could be explained by non-uniform protein expression of mutant SOD1 in various tissues. Studies in human, as well as the SOD1-G93A and SOD1-G37R mice, have demonstrated that SOD1 protein levels vary
between tissues. In SOD1-G93A and SOD1-G37R mouse model, SOD1 protein levels were found to be higher in spinal cord compared to hindlimb skeletal muscle (GA) and liver (Gajowiak et al., 2015; Hilton et al., 2016). Moreover, it was reported that CNS tissues of SOD1-G37R mice expressed much higher amounts of SOD1 compared to PNS tissues (i.e. kidney, liver and quadriceps) (Hilton et al., 2018). Interestingly, in PNS tissues the upregulation of APP levels was found exclusively in fast twitch muscle fibres of SOD1-G93A transgenic mice (Bryson et al., 2012).

Both APP and APLP2 are required for NMJ formation and maintenance during development (Wang et al., 2005). Also, during NMJ development APP mediates the localised activation of caspase 6 and destruction of excited motor axon terminals through the death receptor 6 (Kuester et al., 2011; Nikolaev et al., 2009). This suggests increased APP expression may have a negative impact on compensatory nerve terminal sprouting and formation of the NMJ, which occurs in response to the initial denervation of muscle fibres in MND. It should be noted that SOD1-G93A transgenic mice on an APP null background exhibit NMJ denervation and delayed motor symptoms (Bryson et al., 2012), supporting the notion that the lack of APP is beneficial by reducing disease progression of SOD1 transgenic mice. Our findings suggest that similar to APP, APLP2 could also have a functional role in MND and APLP2 may be involved in regulating pathological outcomes in the murine model of MND.
4.4 Conclusion

Disease progression can be highly variable between animal models of MND and between studies using the same model. The results of this Chapter improve our understanding of the disease development in the SOD1-G37R mouse model. Importantly, we found that APP and APLP2 expression levels were increased in the spinal cord at the end stage of disease in a sex-dependent manner, but levels in the cerebellum, brain stem and muscles were unchanged. These results suggest a possible role for both APP and APLP2 in the pathological processes of MND. The sex-dependent effects on APP and APLP2 levels may also help to explain some aspects of the sex differences observed in human MND, where the frequency of the disease is significantly higher in males compared to females. However, which pathway and how APP and APLP2 are playing a role in the sex differences of MND pathogenesis remains to be explored.
Chapter 5: Examining the behavioural phenotypes and biochemical changes of SOD1-G37R mice with APLP2 gene deletion

5.1 Introduction
In Chapter 4, we found significant increases in APLP2 and APP protein levels in the spinal cord of SOD1-G37R mice at disease end stage (Figure 4.14). Our results also showed that the significant increase in APP and APLP2 levels in SOD1-G37R are sex-dependent. These findings support previous literature on the role of APP in MND (Bryson et al., 2012; Koistinen et al., 2006) and suggest a possible function for APLP2 in MND pathogenesis. As described in Chapter 1 (Section 1.1.2), age and sex are both established risks factors in MND and they may contribute to the clinical heterogeneity amongst MND subjects (Manjaly et al., 2010). The marked influence of sex-dependent effects in MND is mainly attributable to the incidence, clinical presentation and severity of the disease (McCombe and Henderson, 2010). The proportion of MND in the population is slightly higher in men compared to women with a ratio of 1.5:1 (McCombe and Henderson, 2010; Zoccolella et al., 2008). However, this difference diminishes in post-menopausal women (Blasco et al., 2012), suggesting a possible protective role of estrogens in MND pathophysiology (Manjaly et al., 2010). Moreover, sexual dimorphism in clinical phenotypes have also been described in several studies, and all consistently showed a higher incidence of limb onset in men (Galvin et al., 2017), while bulbar onset is more frequent in women (Chio et al., 2009c; Deliz et al., 2018).

Sex differences are not restricted to human cases, transgenic SOD1 mouse models likewise display sex-based disparities in disease onset, symptom severity and disease progression (Julien and Kriz, 2006). Studies have reported that female SOD1-G93A mice (Alexander et al., 2004; Van Den Bosch, 2011) displayed longer lifespans than their male counterparts due to delayed symptom onset (Choi et al., 2008), however no sex differences were also reported in other studies (Miana-Mena et al., 2005). This variability in disease onset was reported to be associated with genetic background, where a longer lifespan was observed in female SOD1-G93A mice on the C57BL/6 background but not on the B6SJL background (Mancuso et al., 2012). However, conflicting results were
presented in studies that showed longer survival in female mice on the B6SJL background but not for the C57BL/6J background (Heiman-Patterson et al., 2005).

Apart from variability due to the genetic background of the animal models, many studies only consider one sex or often fail to compare both sexes. This may explain why therapeutics that are beneficial in transgenic models have translated poorly into human clinical trials tested on both sexes. Therefore, it should be recognised that sex differences exist in MND and sex comparisons should always be considered an important biological variable from basic to preclinical research. Elucidating the precise molecular factors contributing to the sex differences in MND will be pivotal in our understanding of disease heterogeneity.

In this Chapter, we tested the hypothesis that the lack of APLP2 expression could modulate disease progression in vivo by using the SOD1-G37R transgenic MND model. As sex differences may regulate the disease mechanisms implicated in MND, we also investigated if the effects of APLP2 expression on disease progression in SOD1-G37R are sex-dependent.
5.2 Results

5.2.1 Heterozygous deletion of APLP2 gene reduces reproductive frequency in female SOD1-G37R mice

SOD1-G37R mice were bred with APLP2/- mice to obtain SOD1-G37R mice with the APLP2 gene deletion (homozygous and heterozygous deletion). This was done by initially mating hemizygous SOD1-G37R mice with APLP2/- mice to generate the F1 progeny with an expected 50% frequency for SOD1:APLP2+/− and WT:APLP2+/− (Figure 5.1A).

The second round of breeding was set up by mating the progeny of the F1 mice (intercrossed) to generate the F2 generation. The expected Mendelian ratio in the F2 mice should be; 25% for SOD1:APLP2+/− and WT:APLP2+/− and 12.5% for each of SOD1:APLP2+/+, SOD1:APLP2−/−, WT:APLP2−/− or WT:APLP2+/+ (Figure 5.1A).

All mice were genotyped by PCR using mouse tail genomic DNA with PCR primer sets specific for the SOD1 mutant alleles (Figure 5.1B) and APLP2 alleles (Figure 5.1C) to confirm the presence and/or absence of the corresponding alleles. In the F1 generation, all the genotypes were born at the anticipated Mendelian ratios of 50:50 (data not shown), indicating that heterozygous deletion of APLP2 in SOD1-G37R was not embryonic lethal.
Figure 5.1 Generation of SOD1-G37R mice with APLP2 gene deletion

(A) Breeding scheme to generate the SOD1-APLP2 mouse line. (B) PCR analysis of genomic DNA prepared from mouse tail samples and separated by DNA agarose gel electrophoresis. Mice positive for SOD1 transgene showed two bands: top band at ~324bp representing the endogenous SOD1 transgene and a lower band at ~236bp representing the SOD1 transgene. Mice positive for SOD1 transgene have been labelled as SOD1, and non-transgenic littermates have been labelled as WT. (C) Shown in electrophoresis gel image is PCR product using APLP2 gene primer sets. A ~400bp band was obtained from WT mice (+/+), a 350bp band from APLP2 gene knockout mice (-/-), and both bands from heterozygotes (+/-) was detected from an animal with one WT allele, and one APLP2 knockout allele. PC: positive control, NC: negative control (no genomic DNA), bp: base pairs.
The reproductive performance of the F2 was evaluated by recording the number of mating pairs, the number of litters and pups weaned for the study. From over 34 breeder pair matings, which generated over 61 litters and only 39 litters were viable. Interestingly, the total number of litters and viable pups born from the mating between female SOD1:APLP2+/- with male WT:APLP2+/- was substantially lower than the litters born from male SOD1:APLP2+/- and female WT:APLP2+/- mice (Table 5.1). We noticed the increased death rate in pups born was most likely caused by cannibalism. The sex ratios across litters were similar across all genotypes (Table 5.2).

Table 5.1 Comparison of litter size and viable offspring for different mating pairs

<table>
<thead>
<tr>
<th>Parents</th>
<th>Number of pairs</th>
<th>Number of litters</th>
<th>Number of viable litters</th>
<th>Number of viable pups</th>
</tr>
</thead>
<tbody>
<tr>
<td>♀ WT:APLP2+/- × ♂ SOD1:APLP2+/-</td>
<td>26</td>
<td>60</td>
<td>39</td>
<td>239</td>
</tr>
<tr>
<td>♀ SOD1:APLP2+/- × ♂ WT:APLP2+/-</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5.2 Frequency of F2 viable offspring

<table>
<thead>
<tr>
<th></th>
<th>Expected (%)</th>
<th>Observed (%)</th>
<th>Observed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female/Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>SOD1:APLP2+/-</td>
<td>12.50</td>
<td>15.50</td>
<td>14.75</td>
</tr>
<tr>
<td>WT:APLP2+/-</td>
<td>12.50</td>
<td>17.00</td>
<td>10.07</td>
</tr>
<tr>
<td>SOD1:APLP2+/+</td>
<td>6.25</td>
<td>5.00</td>
<td>10.43</td>
</tr>
<tr>
<td>SOD1:APLP2-/-</td>
<td>6.25</td>
<td>4.50</td>
<td>5.04</td>
</tr>
<tr>
<td>WT:APLP2+/+</td>
<td>6.25</td>
<td>6.50</td>
<td>4.68</td>
</tr>
<tr>
<td>WT:APLP2-/-</td>
<td>6.25</td>
<td>1.50</td>
<td>5.04</td>
</tr>
</tbody>
</table>
5.2.2 APLP2 gene deletion reduces body weight of male SOD1-G37R mice

Disease progression of MND can be characterised and monitored by progressive weight loss. To examine the effect of APLP2 expression on the body weight of SOD1-G37R mice, body weight was monitored twice weekly from 6 weeks of age. No significant difference in body weight was observed between the female groups. Male SOD1:APLP2-/- exhibited a reduction in body weight when compared with male SOD1:APLP2+/+ and was significant at 8, 12, and 16 weeks of age (p<0.05). Overall, male mice are significantly heavier than female mice for all SOD1:APLP2 genotypes at any time point (Figure 5.2).

Figure 5.2 APLP2 gene deletion causes a reduction in body weight of male SOD1-G37R mice.

Body weight (g) of SOD1:APLP2+/+, SOD1:APLP2-/- and SOD1:APLP2+/- for (A) female (B) male mice from 6 weeks of age until end-stage of disease. Comparing body weight between female and male groups for (C) SOD1:APLP2+/+, (D) SOD1:APLP2-/- and (E) SOD1:APLP2+/- mice with bar graphs representing the mean body weight across the weeks for each group. Values represented as mean ± SEM, analysed using two-way ANOVA for comparison genotype comparison and by Student t-test for sex comparison, *p<0.05, ****p<0.0001, N=12-16.
5.2.3 APLP2 gene deletion improves survival and lifespan of female SOD1-G37R mice

We determined if APLP2 expression affected lifespan by evaluating their survival rate. The maximum lifespan of female SOD1:APLP2/- was significantly extended as compared to SOD1:APLP2+/+ and SOD1:APLP2+/- (p<0.001, Fig 5.3A) mice groups. No differences were observed between female SOD1:APLP2+/+ and SOD1:APLP2+/-, or between the three male groups (Figure 5.3A-C). Although there was a delayed onset in female SOD1:APLP2+/+ there was no sex-dependent difference in the lifespan between male and female SOD1:APLP2+/+ mice cohort (Figure 5.3D).

For comparison between sex, deletion of APLP2 significantly extended the lifespan of female SOD1-G37R by ~1.5 weeks compared to male SOD1-G37R (p=0.0134, Figure 5.3E). In contrast, heterozygous deletion of APLP2 significantly reduced the lifespan of female SOD1-G37R by ~2 weeks (p=0.092, Figure 5.3F). Collectively, these results indicated SOD1-G37R lifespan is affected by the expression of APLP2 in a sex-dependent manner.

Figure 5.3 APLP2 deletion improves life span of female SOD1-G37R mice. Survival curves for (A) female and (B) male groups and (C) the average survival for SOD1:APLP2+/+, SOD1:APLP2+/- and SOD1:APLP2/- mice groups. Survival curves with log-rank (Mantel-Cox) test were compared between sexes for (D) SOD1:APLP2+/+, (E) SOD1:APLP2+/- and (F) SOD1:APLP2/- mice. Values presented as mean ± SEM, *p<0.05, ***p<0.001, two-way ANOVA with Tukey’s post-hoc test, N=14-27.
5.2.4 APLP2 gene deletion delays motor dysfunction in female SOD1-G37R mice

Motor performance was assessed using an accelerating rotarod in mice beginning at 8 weeks of age to elucidate the sex differences in the survival rate (Figure 5.4). Both female SOD1:APLP2/- and SOD1:APLP2+/+ displayed a significant reduction in motor performance compared to SOD1:APLP2+/+ mice at 22 weeks and 24 weeks of age (p<0.01, Figure 5.4A). Female SOD1:APLP2/- mice showed a delay in acquiring the deficit in rotarod performance compared SOD1:APLP2+/+ mice. In contrast, no significant differences in rotarod performances were observed between the male groups (Figure 5.4B).

Comparing the rotarod data between male and females for each genotype showed that all the male genotypes displayed an earlier deficit in rotarod performance compared to females (Figure 5.4C-E). The rate of deterioration was more aggressive in males, suggesting a more rapid disease progression in males than in females. The SOD1:APLP2+/+ female and male mice displayed similar performance in the rotarod test.

Visual inspection of the rotarod performance curve doesn’t allow us to exactly pinpoint the age of decline in rotarod performance between the animal groups, therefore we opt for a more sophisticated analysis using the GenStat Statistical analysis package. The split line regression model, which splits the data into two regression line representing two phases: the first phase when the animal performs at equilibrium or at an incline in the rotarod performance, while the second phase depicted when the animal showed decline in their performance. The model then detects the precise regression breaks between the two regression lines allowing us to estimate the age of decline in the rotarod performance of each animal.

We found that motor impairment was significantly delayed in onset by ~3 weeks in female SOD1:APLP2 +/+ mice (p<0.05) compared to male mice. Although rotarod performance was lower in female SOD1:APLP2/- mice, motor performance deficits occurred much earlier in the male (~ 6 weeks) compared to female SOD1:APLP2/- mice (p<0.0001). In contrast, male SOD1:APLP2+/+ displayed ~3 weeks delay in rotarod
decline compared to female mice (Figure 5.4F). These results suggest that the improvement in survival in the female SOD1:APLP2/- mice may be associated with the improvement in motor function.

Figure 5.4 Motor performance dysfunction is delayed in female SOD1-G37R mice with APLP2 gene deletion.
Motor function was assessed using the rotarod assay and recorded as latency to fall for (A) female and (B) male SOD1:APLP2+/+, SOD1:APLP2/- and SOD1:APLP2+- mice. Sex comparison for (C) SOD1:APLP2+/+, (D) SOD1:APLP2/- and (E) SOD1:APLP2+/- mice cohorts. F) The age of decline (weeks) for rotarod performance was calculated using the split line regression model in GenStat analysis software. Data are shown as mean ± SEM, analysed using two-way ANOVA with Tukey’s post-hoc test for comparison of rotarod performance across the weeks and by Student t-test for sex comparison using the split line regression model, *p<0.05, **p<0.01, ****p<0.000, N=13-24.
5.2.5 APLP2 gene deletion improves neurological function score in female SOD1-G37R mice

The effect of APLP2 deficiency on disease severity was examined by neurological function score based on the ALSTDI criteria (described in Chapter 2 section 2.3.2). When suspended by the tail, SOD1:APLP2+/+ , SOD1:APLP2+/- and SOD1:APLP2-/- mice at the early disease stage (~14 weeks) exhibited mild hindlimb tremor but could extend their limbs similar to WT:APLP2+/+ and WT:APLP2-/- mice. However, as the disease symptoms developed, hindlimb extension was impaired in all SOD1:APLP2 transgenicas indicated by the progressive increase in the neurological scores for both female and male mice groups (Figure 5.5), suggesting motor neurone disease progressed similarly in these mutant mice. When neurological scores were analysed for each age group we found a significant decrease in the neurological scores of female SOD1:APLP2-/- at 18 weeks of age when compared with both SOD1:APLP2+/+ and SOD1:APLP2+/+ mice for both left and right hindlimbs.

Figure 5.5 Symptoms onset determined by neurological function scores is delayed in female SOD1-G37R mice with APLP2 gene deletion

The effect of APLP2 deletion on the neurological function scores of SOD1 mice from 8 weeks of age until disease end stage for (A) female and (B) male groups of SOD1:APLP2+/+ , SOD1:APLP2-/- and SOD1:APLP2+/+ mice for the (i) left and (ii) right hindlimbs. Values presented as mean ± SEM, two-way ANOVA with Tukey’s post-hoc test for genotype comparison *p<0.05, **p<0.05, N=10-19.
Male SOD1:APLP2-/- mice also exhibited an improvement in the neurological scores, but only in the right hindlimbs at the later age of 24 weeks (p<0.05). Consistent with the rotarod test, a sex comparison of the SOD1:APLP2+/+ showed a significant improvement in neurological function in females at 22 weeks for right hindlimbs. The delay in symptoms onset was more pronounced for SOD1:APLP2-/-, which was extended by ~4 weeks in females to reach a neurological score of 1 (p<0.01, Figure 5.6). No differences were observed for the SOD1:APLP2+- mouse group when sexes were analysed separately for either left or right hindlimbs (Figure 5.6). In summary, the deletion of APLP2 delays disease onset and progression in SOD1-G37R mouse model.
Figure 5.6 Female SOD1-G37R mice with APLP2 gene deletion showed delayed neurological function score compared to male mice. Neurological function scores compared between the female and male cohorts for (A) SOD1:APLP2+/+, (B) SOD1:APLP2-/-, and (C) SOD1:APLP2+/- for the (i) left and (ii) right hindlimbs. Values presented as mean ± SEM, two-way ANOVA with Tukey’s post-hoc test, *p<0.05, **p<0.05, N=10-19.
5.2.6 APLP2 gene deletion in SOD1-G37R mice improves motor performance in female mice prior to symptom onset as detected by DigiGait

Disease progression in MND can be characterised by gait deficits. Therefore, we used the DigiGait platform to investigate if APLP2 expression affects gait dynamics in SOD1:APLP2 and WT:APLP2 mouse lines. It was noted that not all the SOD1:APLP2 mouse lines were able to perform on the DigiGait platform through to disease end stage. Of the three tested speeds examined (10cm/s, 15cm/s and 20cm/s) only the 15cm/s data are shown since this speed allowed both early detection and evaluation of deficits until the late phase of disease.

All SOD1:APLP2 mouse lines were able to perform on the DigiGait platform at 15cm/s from 7 to 12 weeks of age. However, performance rate of SOD1:APLP2+/+ was significantly reduced at 18 weeks of age when only 63.6% (7 of 11) of the female mice and 69.2% (9 of 13) of male mice were able to perform at this speed (Figure 5.7, Kaplan-Meier test, p<0.0001). A comparison between the different genotypes revealed that male SOD1:APLP2-/- mice exhibited a significantly lower performance rate (Log-rank Mantel-Cox test, p<0.05) compared to SOD1:APLP2+/+, but no differences were observed in the female SOD1:APLP2-/- group (Figure 5.7).

![Figure 5.7](image_url)

**Figure 5.7** Male SOD1-G37R mice lacking APLP2 perform worse on the DigiGait analysis

*Performance rate of (A) female and (B) male SOD1:APLP2+/+ (N=11, 13) SOD1:APLP2-/- (N=10, 10) and SOD1:APLP2+/-(N=14, 11) mice respectively on the DigiGait analysis at 15cm/s beginning from 7 weeks of age, measured using log-rank Mantel-Cox test*
To examine the gait dynamics a detailed analysis of more than 50 different gait parameters were evaluated at 15cm/s for both sexes. Similar gait pattern and body movement were observed between the different male SOD1:APLP2 groups (data not shown). Similarly, we also detected gait patterns for the left and right fore-limbs of the female mice cohort. However, a significant difference was observed in the left and right hindlimbs of the female groups for stride length, swing, brake and propel, therefore only hindlimb data of the female groups are shown.

We observed a significant improvement in stride length for both the left and right hindlimbs of female SOD1:APLP2/- mice beginning at 11 weeks of age, and it remained higher than female SOD1:APLP2+/+ as the disease progressed (Figure 5.8). Female SOD1:APLP2+/+ also exhibited a significant improvement in stride length, however, this was significant at a later time point at 12 and 18 weeks of age. Significant improvement in swing, brake and propel indices were also found at 11 weeks for SOD1:APLP2/- and at 12 weeks of age for SOD1:APLP2+/+ mice. These data are consistent with the neurological scores indicating that a lack or reduction in APLP2 expression improves gait function in female SOD1-G37R mice.
Figure 5.8 APLP2 deletion improves various gait indices in the hindlimbs of female SOD1-G37R mice prior to symptom onset

DigiGait analysis of (A) left and (B) right hindlimbs for (i) stride length (cm), (ii) swing (secs), (iii) stance (secs), (iv) brake (sec) and (v) propel (secs) for female SOD1;APLP2+/+, SOD1;APLP2-/- and SOD1;APLP2+/- mice from 7-25 weeks of age with at 15cm/s. Values presented as mean ± SEM, analysed and compared to SOD1:APLP2+/+ using two-way ANOVA with Tukey’s post-hoc test, *p<0.05, **p<0.01, ****p<0.0001, N=5-8.
5.2.7 APLP2 gene deletion in SOD1-G37R mice does not affect motor neuron survival

We evaluated whether the delay in motor function in female SOD1:APLP2-/- mice is associated with the extent of motor neuron degeneration in the spinal cord. Histological examination assessed by Nissl staining of α-motor neurons with a diameter greater than 20cm revealed a significant decrease in α motor neurons in all SOD1 transgenic mice compared to their WT littermate controls (Figure 5.9A). There were no differences in the number of α motor neurons in SOD1:APLP2-/- and SOD1:APLP2+/+ when compared to SOD1:APLP2+/+ for both female and male groups. While there was no difference in motor neuron number between WT:APLP2+/+ and WT:APLP2-/- for the female groups, the male groups displayed a significant ~29.1% reduction (p<0.05) for WT:APLP2-/- mice group. This was confirmed when we compared sex differences for each genotype and found a significant reduction (~33.7%) in α neurons in male WT:APLP2-/- compared to female WT:APLP2-/- mice, but no differences were observed in the other genotypes (Figure 5.9B). These results suggest that APLP2 deficiency in SOD1-G37R does not affect motor neuron viability in this model and that the observed improvement in the survival of female SOD1:APLP2-/- mice is not a result of motor neuron cell body rescue.
Figure 5.9 Motor neuron survival is not rescued in SOD1-G37R mice with the deletion of APLP2 gene.

(A) Representative micrographs of motor neurons in the ventral horn of the spinal cord of female and male cohorts for WT:APLP2+/+, WT:APLP2-/-, SOD1:APLP2+/+, SOD1:APLP2-/- and SOD1:APLP2+/+ mice at disease end stage stained with cresyl violet. Scale bar = 100μm. (B) Bar graphs showed the number of spinal neurons in 7 sections with diameter >20μm in the ventral horn between L4 and L5 spinal cord segments of female and male mice. Values represented mean ± SEM, one-way ANOVA with Bonferroni’s post-hoc test were used for genotype comparison to respective female and male WT:APLP2+/+ mice groups, student t-test, was used for comparison between sexes, *p<0.05, **p<0.01 and ****p<0.0001, N=5-6.
5.2.8 APLP2 gene deletion does not improve astrogliosis in SOD1-G37R mice

Astrogliosis is a pathological feature of MND both in animal models and MND patients (Hall et al., 1998; Kushner et al., 1991), therefore the influence of APLP2 expression on the activation state of astrocytes was examined by quantifying GFAP immunoreactivity levels in spinal cord sections at disease end-stage (Figure 5.10A). The percentage change in the GFAP was significantly higher in the female SOD1:APLP2+/- and male SOD1:APLP2+/- groups when compared with their WT:APLP2+/+ littermate controls (Figure 5.10B). A similar results were observed for both the SOD1:APLP2-/- and SOD1:APLP2+/- mice groups. Sex-dependent comparison of GFAP expression in the different genotypes revealed a significant reduction in the female groups of both SOD1:APLP2+/- and SOD1:APLP2+/- mice. However, no difference in GFAP expression levels were observed between SOD1:APLP2-/-, SOD1:APLP2+/- and SOD1:APLP2+/- mice for both male and female cohorts (Figure 5.10). These results show that genetic deletion of APLP2 does not affect the activation of astrocytes in SOD1-G37R mice.
Figure 5.10 Effects of APL2 expression in SOD1-G37R mice on the activation of astrocytes.

(A) Representative immunohistochemical images showing GFAP+ cells in the ventral horns of L4-L5 segments of male groups of WT:APLP2+/+, WT:APLP2−/−, SOD1:APLP2+/+, SOD1:APLP2−/− and SOD1:APLP2+/− mice at end-stage of the disease. Scale bar = 100μm. (B) Graphs showed the fold change in the percentage fold change of areas positively immune-stained with GFAP normalised to the respective female and male WT:APLP2+/+ mice groups. Values represented mean ± SEM, one-way ANOVA with Bonferroni’s post-hoc test were used for genotype comparison to WT:APLP2+/+, student t-test was used for comparison between sexes, *p<0.05, ***p<0.001 and ****p<0.0001, N=5-6.
5.2.9 APLP2 gene deletion does not improve microgliosis in SOD1-G37R mice

Microglial activation, which parallels MND progression (Hall et al., 1998), was examined in the ventral horn of the spinal cord. Microglia activation, as assessed by quantifying IBA1 immunoreactivity levels in the ventral horn of the spinal cord at disease end stage. The percentage change in IBA1 stained area was significantly increased in the SOD1:APLP2+/+ mice for both female and male (p<0.05) groups compared to the WT:APLP2+/+ control (Figure 5.11). Microglia activation was significantly elevated in both the female and male (p<0.01) groups of SOD1:APLP2-/- mice.

Although we observed a significant increase in IBA1 in the female group of SOD1:APLP2+/+ (p<0.05), the levels of IBA1 in the male group were unchanged. There was no difference in the percentage area of microgliosis between SOD1 mice with different APLP2 alleles for either sex. In all genotypes, sex comparison also showed no differences in the level of IBA1 immunoreactivity (Figure 5.11). This suggests APLP2 expression does not affect microgliosis in SOD1-G37R mice.
Figure 5.11 APLP2 gene deletion does not improve microgliosis in the spinal cord of SOD1-G37R mice
(A) Representative micrographs of the ventral horn lumbar spinal cord immunolabelled with anti-IBA1 antibody from WT:APLP2+/+, WT:APLP2-/-, SOD1:APLP2+/+, SOD1:APLP2-/- and SOD1:APLP2+/- female and male mice at end stage immunohistochemically labelled with anti-IBA1 for microglia expression. Scale bar = 100μm. (B) Graphs showed the percentage fold change for area positive for IBA-1 normalised to the respective male and female WT:APLP2+/+ mice groups. Data represent mean ± SEM, one-way ANOVA with Bonferroni’s post-hoc test were used for genotype comparison to WT:APLP2+/+, student t-test was used for comparison between sexes, *p<0.05, **p<0.01 and ****p<0.0001, N=5-6.
5.2.10 APLP2 gene deletion exacerbates NMJ denervation in male SOD1-G37R mice but improves NMJ innervation in female SOD1-G37R mice

To examine whether the delay in disease onset and survival of female SOD1:APLP2-/- mice relates to the NMJ we analysed the integrity of the NMJ at end-stage of disease using immunofluorescence. NMJs were examined in the GA muscle, and colocalization between alpha-bungarotoxin (α-BTX) and synaptophysin (syn) was assessed. At disease end-stage we observed substantial NMJ denervation in all SOD1:APLP2 mouse lines compared to WT:APLP2 littermates control, as evidenced by the number of NMJs stained with α-BTX lacking presynaptic staining with synaptophysin (Figure 5.12).
Figure 5.12 Defective synapses in the NMJ of SOD1:APLP2 mice at disease end stage
The NMJ in the tibialis muscle sections were identified by immunofluorescence staining using a presynaptic marker, α-Bungarotoxin (green) to label acetylcholine receptors and postsynaptic marker synaptophysin (red) to label nerve terminals, and DAPI (blue) to identify cell nuclei. Staining showed severe morphological changes at the NMJ in the tibialis muscles from end-stage animals of SOD1:APLP2+/+, SOD1:APLP2+-, and SOD1:APLP2-- compared to WT animals. Scale bar = 20μm.
Based on their innervation of the motor end plate, NMJs were classified as fully innervated, partially innervated or denervated junctions. Denervation of NMJs are revealed by a reduction in the number of pretzel shaped NMJs (Chapter 4) are accompanied by the collapse and fragmentation of NMJs structures (Figure 5.13A). Quantification of NMJs in SOD1:APLP2 mouse lines revealed a significantly higher number of denervated NMJ in the male SOD1:APLP2-/- mice with only 11.0 % of NMJs retaining their normal innervated morphology at end-stage (Figure 5.13B). Conversely, the proportion of innervated NMJ was significantly increased in the female SOD1:APLP2-/- mice and only ~43.4 % of junctions were denervated as opposed to ~68.6% observed for SOD1:APLP2+/- mice (p<0.05). No significant differences in the proportion of innervated and denervated junctions were observed in SOD1:APLP2+-/- for either sex.

Sex-based evaluation of the NMJ innervation pattern revealed a significant ~3 fold reduction in fully innervated junctions in female SOD1:APLP2+/- with a significant increase in denervated NMJs in the female mice (Figure 5.13E). Similarly, a sex comparison of NMJ occupancy also showed a significant increase in the denervated junction in female SOD1:APLP2+-/- cohort (Figure 5.13F). In contrast, denervated NMJs were significantly lower in female SOD1:APLP2-/- cohort. NMJ innervation was also improved in the female group compared to male SOD1:APLP2-/- cohort.

Taken together, the results indicate the delay in disease onset and survival in SOD1-G37R:APLP2-/- could be driven by a reduction in synaptic dysfunction, and these synaptic changes are sex-dependent.
Figure 5.13 APLP2 gene deletion ameliorates NMJ denervation in female SOD1 mice.

(A) Representative images of colocalization between pre-synaptic and post-synaptic marker defined as fully innervated, partially innervated and denervated NMJ. Arrows indicated collapsed and fragmented NMJ. Calculated percentages of fully innervated NMJ (>20% colocalisation), partially innervated NMJ (10-20% colocalisation) and denervated NMJ (<10% colocalisation) in three muscle sections (100µm apart) for (B) male and (C) female SOD1:APLP2+/+, SOD1:APLP2/- and SOD1:APLP2+- mice groups. NMJ innervation status were also compared between sexes for (D) SOD1:APLP2+/+, (E) SOD1:APLP2/- and (F) SOD1:APLP2+- mice groups. Data represented mean ± SEM, one-way ANOVA with Bonferroni’s post-hoc test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, N=3-4. Scale bar=10µm.
5.2.11 APLP2 gene deletion exacerbates muscle atrophy in male SOD1-G37R mice but ameliorates atrophy in female SOD1-G37R mice

To investigate whether the deteriorating locomotor function in SOD1:APLP2 mice coincides with progressive and widespread wasting of hindlimb muscles, transverse sections of gastrocnemius muscle from SOD1:APLP2 mice were examined with an ATPase stain. The muscle myofibre cross-sectional area was measured, counted and binned into separate groups according to their size. In contrast to the WT:APLP2 controls which showed an even staining pattern of type I and type II myofibres, the muscle morphology of SOD1:APLP2 mouse lines at end-stage was disrupted and displayed a shrunken appearance (Figure 5.14A). Overall, morphological analysis showed that SOD1:APLP2 mice groups showed an increased centrally localized nuclei muscle cells compared to WT:APLP2 mice groups. During nerve damage or synaptic dysfunction, muscle fibres can be degenerate and in the attempt to counteract the functional loss new muscle fibres can be replaced therefore a higher rate of muscle fibres turnover may be seen (Schmalbruch, 1976).

Quantification of the CSA of the type I slow twitch muscles showed no change in SOD1:APLP2/- and SOD1:APLP2+/− when compared with SOD1:APLP2+/+ mice for either sex (Figure 5.14B-D). In contrast, analysis of CSA of fast twitch type II muscle fibres, the most vulnerable fibres in MND pathogenesis (Frey et al., 2000; Pun et al., 2006; Vinsant et al., 2013), showed a significant ~40.0% reduction in male SOD1:APLP2/- compared to both SOD1:APLP2+/+ and SOD1:APLP2+/− mouse groups (Figure 5.14E). This confirms that the loss of muscle mass of GA (data not shown) is predominantly based on type II fiber atrophy and explains why GA muscle showed the most decline in muscle mass, being composed almost exclusively of type II muscle fibres.

The CSA of fast twitch fibres were significantly higher in the female SOD1:APLP2/- compared to both SOD1:APLP2+/+ and SOD1:APLP2+/− mice (Figure 5.14F). While no changes were observed in the CSA of fast twitch fibres for male SOD1:APLP2+/−, a significant reduction in CSA was observed for female SOD1:APLP2+/− when compared with SOD1:APLP2+/+ mice. The reduced NMJ denervation in the female SOD1:APLP2/- mice may help explain why female SOD1:APLP2/- mice showed improved muscular atrophy and hence improved motor function compared to male SOD1:APLP2/- mice.
Myofibre quantification showed that only female SOD1:APLP2 +/- mice showed a significant increase in the number of fast twitch myofibres compared to female WT:APLP2+/+ mice (~45.0%), and no differences were observed for the other groups (Figure 5.14G). Sex comparison of CSA across the different genotypes revealed a significant reduction in CSA in female groups of SOD1:APLP2+/+ and SOD1:APLP2+- (Figure 5.14H-J) for type II myofibres (Figure 5.14I). These results parallel the NMJ results and indicate APLP2 deficiency affects lower motor neuron pathology in SOD1-G37R mice.
Figure 5.14 Genetic deletion of APLP2 expression in female mutant SOD1-G37R mice improves muscle fibre atrophy.

(A) Representative micrographs of gastrocnemius muscle cross-sections stain with ATPase (pH 4.3) from female and male SOD1: APLP2+/+, SOD1:APLP2-/-, SOD1:APLP2+/− at the end stage of disease and their age-matched WT littermates control WT:APLP2+/+ and WT:APLP2-/. Quantification of (B-D) slow twitch/type I and (E-G) fast twitch/type II myofibres cross-sectional area and myofiber count for genotype comparison within the same sex and (H-J) sex comparison within the same genotype. Data represent mean ± SEM, one-way ANOVA with Bonferroni’s post-hoc test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, N=4-6. Scale bar = 100μm.
5.2.12 APLP2 deficiency downregulates APP protein expression in SOD1-G37R mice

We used western blotting to investigate if APLP2 deficiency in SOD1-G37R mice affected the expression of APP. We examined the brain, spinal cord and hindlimb muscle of SOD1:APLP2 mice at end-stage of disease and compared APP levels to the WT:APLP2 littermates.

We observed no changes in the expression levels of APP in the brain of SOD1:APLP2 mice for either sex (Figure 5.15 A-B). However, a significant increase ~2.5-3.5 fold in APP expression was observed in the spinal cord of all SOD1:APLP2 genotypes for both female (p<0.05) and male (p<0.05) groups (Figure 5.15 C-D).

A significant increase in expression of the ~87kDa band in the hindlimb muscle was only detected in female SOD1:APLP2-/- (~5.3 fold) and male SOD1:APLP2+/+ (~4.8 fold) when compared to WT:APLP2+/+ mice. Other APP fragments with molecular weights of ~55kDa and ~25kDa were also detected in the hindlimb muscle but these were not significantly different (Figure 5.15 E-H). No detectable differences were observed between SOD1:APLP2-/- and SOD1:APLP2+/- when compared with SOD1:APLP2+/+ for all these tissues in both sexes.

Although western blot data showed no differences in APP expression levels between the female and male groups in all SOD1:APLP2 genotypes. When we examined the relative expression of APP in the ventral horn of the lumbar spinal cord using immunohistochemistry, we observed a significant decrease in the levels of APP in the SOD1:APLP2-/- (female; ~50.2%, male; ~20.0%) and SOD1:APLP2+/+ (female; ~47.5%, male; ~27.4%) for both sexes when compared to the respective female and male SOD1:APLP2+/+ mice. A sex comparison showed a significant reduction in the levels of APP in the spinal cord of female SOD1:APLP2-/- (~39.6%, p<0.05) and female SOD1:APLP2+/+ (~28.1%, p<0.05 but no sex difference was observed in the SOD1 controls (Figure 5.16). In summary, these results indicate the lack or a reduction in APLP2 gene expression decreased the protein levels of APP in the SOD1-G37R transgenic mice.
Figure 5.15 APP expression is increased in the spinal cord and muscle of SOD1-G37R.
Representative immunoblots and densitometric quantification of APP in the (A, B) spinal cord, (C, D) brain and (E-H) hindlimb muscles (detected at ~87kDa, 55kDa and 25kDa) of SOD1:APLP2+/+, SOD1:APLP2+/−, SOD1:APLP2−/− mice at disease end stage and WT:APLP2+/+, WT:APLP2−/− age-matched littermate controls. For each respective tissues APP−/− and WT:APLP2+/+ mice were used as negative and positive controls respectively. APP levels were normalised to their respective male and female WT:APLP2+/+ mice groups respectively. Values represented mean ± SEM, one-way ANOVA with Bonferroni’s post-hoc test, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared to WT:APLP2+/+, #p<0.05 and ##p<0.01 compared to WT:APLP2−/− mice.
Figure 5.16 Genetic deletion of APLP2 gene decreases APP expression levels in the ventral horn of the lumbar spinal cord of SOD1-G37R mice. 

(A) Immunohistochemical micrographs of the lumbar spinal cord labelled with anti-APP (22C11) antibody for SOD1:APLP2+/+, SOD1:APLP2−/−, SOD1:APLP2+/− mice at end stage of disease and their age-matched WT:APLP2+/+ and WT:APLP2−/− littermates control. Scale bar = 100µm. 

(B) Quantification of the relative pixel intensity of APP expression levels of the female and male groups normalised to their respective WT:APLP2+/+ mice groups. Data represent mean ± SEM, one-way ANOVA with Bonferroni’s post-hoc test were used for genotype comparison to respective female and male WT:APLP2+/+ mice groups, student t-test, was used for comparison between sexes, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, N=4-6.
5.2.13 Identification of sex hormones transcription binding sites in APLP2 promoter sequence

Sex bias has been reported in the development and progression of psychiatric condition such as schizophrenia (Abel et al., 2010; Cowell et al., 1996) and neurodegenerative diseases like Parkinson’s disease (Jurado-Coronel et al., 2018), Huntington’s disease (Zielonka et al., 2013) and Alzheimer’s disease (Miech et al., 2002). These studies highlight the potential influence of sex hormones such as estrogen, progesterone and androgens on disease prevalence, severity and manifestations (Gurvich et al., 2018; Vest and Pike, 2013).

Estrogens play an important role in the vertebrate reproductive system, particularly in the development of the female sex organs and their function (Heldring et al., 2007). The bulk of estrogen signalling is controlled by estrogen receptors (ER) α and β, and both members bind to the estrogen response element (ERE) on the promoters of the genes that they regulate (Heldring et al., 2007; Klinge, 2001). The full ERE motif is 13 bp in length and have the consensus sequence 5’-GGTCA•••TGACC-3’ (Driscoll et al., 1998). The biological actions of progesterone are mediated through two progesterone receptors (PR) that consist of two isoforms PR-A and PR-B (Kastner et al., 1990). The consensus sequence for progesterone response element (PRE) is 5’-G•ACA• ••TGT•C-3’ (Lieberman et al., 1993). The androgen receptor (AR) is a ligand activated transcription factor, known to influence the expression of its target genes by binding to different sets of androgen response elements (ARE) plays a central role in male sexual development (Tan et al., 2015). There is a wide range of sequences that AR can bind, typically containing the core requirement of three out of four guanines contacts with the consensus sequence 5’-GGTACA•••TGTCT-3’(Roche et al., 1992).

We utilised an in-silico approach to explore whether the APLP2 associated sex-dependent effects could be mediated by sex hormone response elements in the APLP2 promoter. Using PROMO (version 8.3), a search engine to predict transcription factor binding sites in DNA sequences, we analysed both the mouse and human APLP2 gene promoter sequences to identify sex hormones regulatory motifs. Analysis of 3.0kb 5’ upstream genomic sequence showed the presence of multiple PRE (24, 24), ERE (4, 5) and ARE (10, 6) sites on both mouse and human APLP2 gene respectively (Figure 5.17). We
identified the presence of PR-A and PR-B (respectively) isoforms for progesterone on both the mouse and human APLP2 sequences. While the presence of both ER-α and -β isoforms was identified in mouse APLP2 sequence, only ER-α were identified in the human APLP2 sequence. These results identify the presence of sex hormone regulatory motifs in the APLP2 promoter regions and support this as a possible mechanism to explain the APLP2 sex-dependent effects in SOD1-G37R pathogenesis.

Figure 5.17 Sequence analysis of mouse and human APLP2 promoter region shows the presence of sex hormones transcription factors binding sites

Schematic representation of APLP2 promoter region for (A) mouse (NC_000075.6) and (B) human (NC_000011.10) APLP2 gene. Shown are the location of potential transcription factor binding sites along the 3.0kb promoter region upstream sequence of mouse and human APLP2 genome for progesterone response element (PRE), estrogen response element (ERE), and androgen response element (ARE).
5.3 Discussion

In this Chapter, we evaluated the role of APLP2 in the SOD1-G37R mouse model of MND. We found that the lack of APLP2 expression exerted beneficial effects on muscle function, attenuated NMJ denervation, reduced muscle atrophy, ameliorated motor performance decline and extended survival of SOD1-G37R mice in a sex-dependent manner.

The triggering event that causes motor neuron degeneration in MND is still largely debated (see Chapter 1, Section 1.4). However, it is known that distal axonopathy with NMJ degeneration is seen early in the pathological process of disease in SOD1 mice and this was observed prior to motor neuron death (Durand et al., 2006; Hegedus et al., 2007; Pun et al., 2006; Schaefer et al., 2005). The results from these studies suggest that NMJ dismantling is the trigger that causes motor neuron death. Subsequent studies also showed restricted expression of human SOD1 transgene in mouse skeletal muscle resulted in NMJ dismantling, motor neuron distal axonopathy, motor neuron death and motor deficits (Dobrowolny et al., 2008; Wong and Martin, 2010). This is further supported by studies which showed that SOD1-G93A mice with muscle-restricted expression of IGF-1, a neurotrophic factor, protected NMJs integrity, enhanced motor neuron survival, delayed disease onset and extended survival of mice by ~4 weeks (Dobrowolny et al., 2005).

In SOD1-G93A mice, APP upregulation was found to occur exclusively in type IIb fibres and this upregulation begins concurrently with NMJ dysfunction participating in muscle fibre atrophy and NMJ denervation (Bryson et al., 2012). Type II fast twitch fibres are mostly innervated by α-motor neurons, the neurons most susceptible to degeneration in MND (Conradi and Ronnevi, 1993; Hadzipasic et al., 2014; Lalancette-Hebert et al., 2016), and are responsible for generating tension via muscle contraction (Burke et al., 1977; Eccles et al., 1960). During NMJ development APP has been reported to function with death receptor 6 to induce neuronal cell death and axon degeneration via the activation of caspase signalling pathways (Nikolaev et al., 2009). This supports the notion that the increase in APP expression in MND (Bryson et al., 2012) may have a negative impact on disease progression, particularly at the NMJ. We also observed a significant increase in APLP2 levels in SOD1-G37R mice with disease development (Chapter 4),
suggesting that APLP2 expression, like APP, may have a negative impact and promote the pathophysiology of MND.

The pathological process occurring at the NMJ and in the muscles may be attributed to the functional roles of APP family in response to MND neuropathology. Both APP and APLP2 are important in NMJ formation and maintenance during development (Klevanski et al., 2014; Wang et al., 2005). The functional roles of APP and APLP2 in MND may be associated with the phenomenon of NMJ degeneration being the triggering event in MND pathogenesis. Our results in this Chapter are in part similar to the results reported for SOD1-G93A:APP knockout model, in which disease progression was delayed (Bryson et al., 2012) with decreased motor neuron death, reduction in NMJ denervation and improved muscle function (Bryson et al., 2012). Despite a decrease in motor neuron death, there was no change in survival (Bryson et al., 2012). It was suggested APP may be contributing to two pathological processes in MND. One pathway involves the upregulation of APP in the CNS, where the amyloidogenic processing of APP contributes actively to motor neuron death, while the other is in the PNS (Bryson et al., 2012).

Furthermore, several studies have demonstrated that preventing motor neuron soma death in MND, via the deletion of genes that controls apoptotic cell death, does not cease disease progression. The deletion of Bax, an important gene involved in the activation of the mitochondrial apoptosis pathway, have been shown to protect motor neuron degeneration against astrocyte mediated toxicity in a model of sporadic MND (Re et al., 2014). Although Bax deletion in SOD1-G93A mice delayed the onset of disease and moderately increased lifespan, it failed to alter disease duration (Gould et al., 2006). This has also been demonstrated previously where the overexpression of anti-apoptotic protein Bcl-2 did not alter disease progression in SOD1-G93A mice (Reyes et al., 2010). Similarly, the double knockout of Bax and Bak (a Bcl-2 homologous antagonist) could substantially delay motor neurons death but it did not restore axonal integrity to the same degree and the motor axon terminals continued to die back from the NMJ (Reyes et al., 2010). These data highlight that preservation of motor neuron soma death alone does not prevent axons from degenerating. Moreover, these studies indicate preservation of the NMJ, and the synapses between motor axons and skeletal muscle are sufficient to delay muscle atrophy.
and would improve muscle function and extend survival in the absence of motor neuron soma death rescue.

The results from our study support APLP2, like APP, to be important in regulating the pathological process of MND in the PNS. The genetic deletion of APLP2 in SOD1-G37R mice significantly decreased NMJ denervation, reduced muscle atrophy, ameliorated muscle function decline and extended survival of SOD1-G37R mice by ~2 weeks. However, we observed no rescue of α-motor neurons or a reduction of neuroinflammation in the spinal cord of these mice, supporting that APLP2 may be contributing to the pathological process of MND in the PNS rather than CNS. While APP and APLP2 share functional similarities at the NMJ in MND pathogenesis, their expression also has distinctly different effects in the CNS and PNS. These data, support the model that APLP2 may be associated with NMJ denervation in MND, a predominant pathological feature of motor dysfunction in MND as proposed in the dying back hypothesis (Fischer et al., 2004; Gould et al., 2006).

Our study, together with the results by Bryson et al. (2012), demonstrated that both APP and APLP2 expression exerts detrimental effects on peripheral denervation during the pathogenesis of MND in different SOD1 mouse models - likely mediated by its actions at the NMJ. Collectively, these studies support skeletal muscle being a primary target for human SOD1 toxicity. It also emphasises the functional importance of NMJ and skeletal muscle in preserving motor function.

APP and its family members are multimodal proteins that can function as cell surface receptor-like proteins or act as ligands to elicit functions (reviewed by (Müller et al., 2017; Thinakaran and Koo, 2008; Zheng and Koo, 2011). Co-binding between APP-family members (homo- and heterodimers in cis and trans orientation) can impact on its processing and function by regulating cell migration and synaptic function (Kaden et al., 2009; Rice et al., 2019; Soba et al., 2005; Wang et al., 2009). Given the functional redundancy between APP and APLPs, as shown in APP-family knockout mouse models (review in Chapter 1, Section 1.5.4) we explored the relationships between APP and APLP2 in the pathogenesis of MND by analysing APP expression levels. Despite the increase in APP expression in SOD1-G37R mice at disease end stage, we observed no change in BACE1 or NRG1 levels in either tissue (Appendix D, Figure D. 1). These
results agree with previous reports that the increase in APP expression in the spinal cords of SOD1-G93A is caused by an increase in APP phosphorylation while BACE1 levels are unchanged (Rabinovich-Toidman et al., 2015). While western blot data showed no change in APP levels at the whole tissue level, immunohistochemistry revealed that the reduction or absence of APLP2 in SOD1-G37R mice caused a significant decrease in APP expression in the ventral horn spinal cord of SOD1-G37R mice. Given the limitations in measuring protein expression quantitatively using immunohistochemistry with DAB, future work looking at the subcellular localisation of APP with neurons, astrocytes and microglia in SOD1:APLP2-/- mice using immunofluorescence would enhance our understanding in the role of APP and APLP2 in MND pathogenesis.

These data have parallels with the analysis of APP and APLP2 expression in cancers where APP is overexpressed in many cancer types including, prostate (Miyazaki et al., 2014), breast (Takagi et al., 2013), colon (Meng et al., 2001), thyroid (Yang et al., 2012), lung (Sobol et al., 2015), pancreas (Hansel et al., 2003) and gastrointestinal tract (Arvidsson et al., 2008). The upregulation of APP in cancer increases cellular growth, differentiation and migration (Hansel et al., 2003; Lim et al., 2014; Venkataramani et al., 2010). Similar to APP, APLP2 is elevated in tumours (Covell et al., 2003), colon cancer (Moss et al., 2007), invasive breast cancer (Abba et al., 2004), pancreatic cancer and metastatic lesions (Peters et al., 2012; Peters et al., 2015).

The relationship between APP and APLP2 in pancreatic cancer has been proposed to involve both molecules contributes to the same growth pathway to activate cellular proliferation (Peters et al., 2012). However, it was suggested they also serve unique roles in pancreatic cancer cell growth since growth inhibition was observed when either APP or APLP2 was downregulated indicating the loss of one protein is not compensated by the expression of the other parologue (Peters et al., 2012). It is possible that in MND, like in cancer, APP and APLP2 act on the same pathway but can serve unique roles in neurons and at the NMJ where APP and APLP2 interact with other molecules to elucidate their function(s).

Apart from MND, the upregulation of APP has also been shown following traumatic brain injury (TBI) both in human and in animal models (Gentleman et al., 1993; Pierce et al., 1996; Van den Heuvel et al., 1999). This upregulation of APP corresponds to a
neuroprotective response (Plummer et al., 2016; Van den Heuvel et al., 1999). The intracerebroventricular administration of sAPPα to rats following TBI decreased axonal injury, reduced apoptosis and improved functional outcomes (Thornton et al., 2006). APP-KO mice. Moreover, APP-KO mice following TBI are more susceptible to injury with poorer motor and cognitive outcomes, increased lesion volume and hippocampal damage compared to injured WT mice (Corrigan et al., 2012a). sAPPα treatment of TBI APP-KO mice rescued these effects, (Corrigan et al., 2012b). The neuroprotective site in APP for TBI was localised to residues 96-110 in a heparin binding site (Corrigan et al., 2011; Corrigan et al., 2014). The current model proposes that upregulation of APP following injury serves as an adaptive and protective response (Corrigan et al., 2012b; Plummer et al., 2016). However, in contrast to the TBI data, the genetic deletion of APP in SOD1 transgenic mice rescued motor neuron death and ameliorated NMJ denervation (Bryson et al., 2012), therefore arguing against neuroprotective roles for APP in MND. It also shows APP can have quite distinct effects depending on the injury model.

The patterns of NMJ susceptibility and resistance observed in both ageing and MND appear to be correlated. A comparison between aged mice (22-28 months of age) and symptomatic SOD1-G93A mice revealed significant similarities in the structural alterations of the NMJs in EDL muscle, including postsynaptic fragmentation, decreased acetylcholine receptor density, NMJ denervation, terminal sprouting, axonal distension and dystrophy (Valdez et al., 2012). Since ageing and MND share similar susceptibility, it is important to evaluate the molecular and functional impact of APLP2 expression on these aspects. In this study, we found that genetic deletion of APLP2 only ameliorated NMJ denervation in female SOD1-G37R mice, but enhanced NMJ denervation in males. Muscle fibre diameter and muscle mass are generally higher in males, while female muscle possesses a higher fatigue resistance than male under submaximal contractions (Miller et al., 1993). However, as previously shown in Chapter 3, the age-dependent and region-specific motor neuron loss seen in WT mice contributes to motor function decline and is unaffected by sex differences. These age-related effects were spared in aged female APLP2-/- mice, suggesting APLP2 mediated effects on motor neuron and muscle morphology occurs in a sex-dependent manner. Collectively, these results suggest the involvement of APLP2 expression in the pathogenesis of MND is sex-dependent, and this is correlated with the detrimental effect of APLP2 on distal axonal pathology in female
mice during ageing. These results highlight that APP and APLP2 expression contribute to the biological mechanisms underlying the importance of sex-dependent differences in MND susceptibility.

Sex differences in disease manifestations, progression and prevalence have been noted in numerous illnesses including autoimmune disease (Ngo et al., 2014), cardiovascular disease (Regitz-Zagrosek and Kararigas, 2017), cancers (Kim et al., 2018a) and neurological disorders (Pinares-Garcia et al., 2018). Sex differences have been reported in MND, with a slightly higher prevalence in men (1.5:1, male to female ratio) (McCombe and Henderson, 2010; Zoccolella et al., 2008). Moreover, epidemiological studies showed male MND patients tend to show an earlier age of disease onset, and this is also recapitulated in selective transgenic animal models of MND (Heiman-Patterson et al., 2005; Heiman-Patterson et al., 2011).

To further elucidate sex differences in the disease phenotype of SOD1-G37R mice in the absence of APLP2 expression, sequence analysis of transcription factors in the promoter region of mouse and human APLP2 gene was performed using the online transcription factor prediction program PROMO. We found multiple occurrences of the consensus sequence for transcription factor binding sites for progesterone, estrogen and androgen on both the mouse and human APLP2 genes. ERα plays a key role in the regulation of the mitochondrial proteostasis response influencing sex differences in SOD1-G93A mice (Riar et al., 2017). The sex-dependent differences in MND have been linked to peroxisome proliferator-activated receptor coactivator 1-alpha (PGC-1α), a master regulator of mitochondrial metabolism (Austin and St-Pierre, 2012; Liang and Ward, 2006). The deficiency of PGC-1α leads to a shortened survival in male but not female SOD1 transgenic mice (Eschbach et al., 2013). PGC-1α has been shown to enhances the transcriptional activities of sex hormone receptors such as AR and ER (Shiota et al., 2010; Tcherepanova et al., 2000). The interaction PGC-1α with AR enhances the DNA binding of AR to ARE, thereby increasing the transcription of AR target genes which promotes prostate cancer cell growth (Shiota et al., 2010). Similarly, PGC-1α is a coactivator of ER-α and regulates the transcriptional activity of ER with target genes (Tcherepanova et al., 2000). It is possible that the significant sex differences seen in the SOD1-G37R model are mediated by sex hormones affecting the expression of APLP2. Whether this is linked
to mitochondrial metabolism and PGC-1α is an avenue worth exploring. Our data provide
a novel pathway linking APLP2 expression with sex-dependent effects in MND that may
explain the sexual dimorphism seen in MND.
5.4 Conclusion
Within the present study, we reported that the expression of APLP2 has a detrimental effect on disease progression in the SOD1-G37R mouse model of MND in a sex-dependent manner. Our study highlights a complex interaction between APP and APLP2 upon MND pathophysiology, and that the APP-family is a relevant target for further investigation into the cause and regulation of MND.

This study also underlines the importance of future studies directed at establishing the role of sex hormones and their related molecules in MND for a therapeutic target.
Chapter 6: Summary and Future Directions

The APP-family has a range of functionally important roles in both neuronal and non-neuronal tissues. Recently, the role of APP in MND pathogenesis was explored, and elevated APP levels in MND patients and transgenic MND mouse models correlated with clinical symptoms (Koistinen et al., 2006). The deletion of the APP gene in a transgenic SOD1 MND mouse model ameliorated disease progression with less motor neuron death and better motor function (Bryson et al., 2012), suggesting a functional role for APP in modulating disease progression in MND.

The studies performed in this thesis has sought to extend this work by examining the key APP-family member APLP2 in MND. We investigated (i) the function of APLP2 and APP expression in motor physiology during ageing and (ii) tested the role of APLP2 in MND disease progression in vivo by generating a SOD1:APLP2-knockout transgenic mouse model.

In Chapter 3 we made several significant and novel observations that extended our understanding of the specific roles of APLP2 and APP in motor function, motor neuron and muscle fibre composition during ageing. We observed a significant reduction in the number of α motor neurons in APLP2-/- mice at 12 weeks of age without an obvious alteration in locomotor function, possibly owing to the compensatory function of other APP family members. Moreover, the motor neuron size distribution is altered in the APLP2 knockout mice, with a significant increase in the smaller sized motor neurons (10-12μm) and a significant decrease in larger sized motor neurons. These results indicated APLP2 expression levels modulate motor neuron size distribution. Interestingly, the reduction in α motor neurons was only achieved in male APLP2-/- mice but reverted to wildtype levels in the 48 week female group. This result is in agreement with the reduction in the CSA of muscle fibre type II for male APLP2-/- at 48 weeks of age, which was unchanged for the female group. The age and sex related differences in motor neuron and muscle size distribution help explain the improvement in motor function observed in female APLP2-/- mice during ageing. We also observed in this Chapter that the genetic deletion of APP expression leads to a reduction of α motor neurons and muscle fibre CSA. These results are in agreement with previous studies where APP-/- mice exhibited
reduced body weight, locomotor activity and forelimb grip strength (Muller et al., 1994; Senechal et al., 2008; Tremml et al., 1998; Tremml et al., 2002; Zheng et al., 1995). Given the roles of APP and APLP2 in the development, maturation and maintenance of NMJ (described in detail in Chapter 1), this may explain the alterations in motor neuron distribution and muscle fibre size in APP-/- and APLP2-/- mice during ageing. Collectively, our data in Chapter 3 enhance our understanding on the functions of APP and APLP2 in motor neuron and muscle function, and it suggests that the actions of APP and APLP2 in the neuromuscular system may be influenced by sex differences.

Although Chapter 3 addresses the effects of APP and APLP2 on motor neuron and muscle function, it doesn’t look at the role of APP and APLP2 on synaptic function. Future studies can examine the structural pattern of NMJ innervation in the muscle in combination with electrophysiological studies by examining the conduction properties of nerve axons in the spinal cord of APP and APLP2 single knockout mice during ageing. This would enhance our understanding of the role of APP and APLP2 in motor neurons and muscle function, and synaptic transmission. Another avenue for future studies will be to examine the compensatory function of APP and APLP1 at the NMJ in the absence of APLP2 expression in mice during ageing. This can be done by examining the expression pattern of APP and APLP1 at the NMJ in APLP2-/- mice during ageing.

In Chapter 4 we investigated the role of APP and APLP2 in MND by examining their expression during disease progression in the SOD1-G37R transgenic mouse model of MND. Due to the large variations in age of onset, disease duration and survival time between different MND SOD1 transgenic mouse models, we first established the phenotype of the SOD1-G37R mouse model used in this study. We also compared both male and females to determine if the disease phenotypes display sex-dependent differences. We observed, using the rotarod and stride length assays, that disease onset was delayed by ~1-2 weeks in female SOD1-G37R mice. No differences in body weight decline, neurological scoring and survival were found between female and male SOD1-G37R mice. These results demonstrated moderate phenotypic sex differences exist in the SOD1-G37R mouse model that agree with the reported differences in human cases of MND (McCombe and Henderson, 2010; Zoccolella et al., 2008).
Based on the disease timeline, the protein expression levels of APP and APLP2 were examined at three timepoints in the SOD1-G37R mice: presymptomatic, symptomatic and end stage of the disease. Both APP and APLP2 protein expression levels were upregulated only in the spinal cord of the SOD1-G37R mice at the end stage of the disease. This supports other studies which showed upregulation of APP in the muscle of both MND patients and SOD1-G93A mice (Koistinen et al., 2006). Interestingly, we found that APP and APLP2 protein expression levels are highly elevated in male SOD1-G37R mice at end stage compared to female mice. These results suggest the upregulation of APP and APLP2 is sex-dependent and may affect the MND disease phenotypes. One of the limitations of the results in this chapter is the insufficient number of animals to allow sex-dependent differences of APP and APLP2 protein levels at the presymptomatic and symptomatic stages of the disease to be statistically assessed. Thus, future work using a larger cohort of animals is needed to examine if there are sex-dependent differences in the expression levels of APP and APLP2 during disease progression in SOD1-G37R mice. Moreover, examining APLP2 expression in human MND cases is needed to understand if the mouse findings translate to the human condition. In addition, future studies using immunohistochemistry can be performed to clarify the cellular and subcellular localisation of APP and APLP2 at the NMJ and motor neurons in MND tissues. This would ascertain whether the changes in APP and APLP2 expression could have a role in NMJ degeneration of MND.

In Chapter 5, we directly tested the role of APLP2 expression in MND by crossing SOD1-G37R mice with APLP2-/- mice to generate the SOD1-G37R:APLP2-/- line. Our results showed that the genetic deletion of APLP2 ameliorates motor dysfunction and improves survival of female SOD1-G37R mice, but there was no difference in the behavioural phenotype of male mice. These results indicate APLP2 expression can modulate disease progression of MND in a sex-dependent manner.

Histological analysis of spinal neurons was conducted as the first step towards understanding the mechanisms that underlie the sex-dependent differences in the behavioural phenotypes. Since APP and APLP2 share overlapping functions and both proteins are increased in MND, we hypothesised the APLP2 gene deletion in SOD1-G37R would also reduce motor neuron death, as described for SOD1:APP/- mice.
(Bryson et al., 2012). However, despite the significant increase in the survival of female SOD1-G37R:APLP2/- mice, we found no significant improvement in motor neuron death or neuroinflammation. These results highlight APP and APLP2 possessing distinct functions and suggest they may affect alternate signalling pathways in the pathophysiology of MND. Surprisingly, further investigations into the biochemical changes at the NMJs revealed that the significant improvement in the denervation pattern and muscle atrophy was reduced in the female SOD1-G37R:APLP2/- when compared to male mice and SOD1-G37R:APLP2 wild-type littermates. Together, these results indicate that the improvement in the disease phenotype and survival of female SOD1-G37R:APLP2/- mice is solely modulated by APLP2 expression affecting periphery synapses in response to the pathophysiology occurring during disease. The novel findings of this thesis extend upon previous work demonstrating the distinct and indispensable function of APLP2 in the formation, maturation and maintenance of NMJs (reviewed Chapter 1, Section 1.5).

APLP2’s effects on the pathological process of MND can be sex-dependent. In Chapter 3, we observed that female APLP2/- mice showed better motor function compared to male APLP2/- mice and WT littermates during ageing. This suggested APLP2 expression in females may exert a partial protective effect on the neurodegenerative changes during ageing or in response to oxidative stress. This is supported by results in Chapter 4 where we observed a sex-dependent elevation of APLP2 levels in the spinal cord of SOD1-G37R at disease end stage, where male mice showed higher APLP2 levels compared to female mice. This would explain why the different female SOD1-G37R genotypes showed a slight delay in disease compared to the male mice. This would also explain why female SOD1:APLP2/- mice had delayed motor dysfunction during disease progression, as well as longer survival (Chapter 5).

Next, we examined whether the APLP2 gene is associated with sex hormone promoter regulatory motifs. Analysis of both the mouse and human APLP2 promoter sequence showed the presence of hormone regulatory motifs with PRE, ERE and ARE sites on both the mouse and human APLP2 genes, suggesting the involvement of sex hormones in regulating APLP2 expression (Chapter 5). To extend these results, future studies would require examining the levels of sex steroid hormone receptors in APLP2/- and
SOD1:APLP2-/ mice, with further investigation into specific timepoint during the disease progression and the influence of sex hormones in muscle. In addition, future work will be needed to test the involvement of sex hormones in regulating APLP2 expression, as it is unclear which sex hormones or pathways APLP2 is involved in to mediate its sex specific effects. Possible studies would include either the use of sex hormone treatment therapy or gonadectomy in SOD1:APLP2-/ mice. These studies would enable more control over sex hormones levels in the circulation to study testosterone, oestradiol and progesterone specific actions and their interaction at different levels.

The deleterious effects of APLP2 expression during MND progression identified in our study is in alignment with previous reports of APLP2 function in cancer metastasis (as discussed in Chapter 5). In cancer, APLP2 levels are upregulated in several cancer types (colon, breast and pancreatic cancer), and the upregulation of APLP2 expression correlated with the aggressiveness of cancer, suggesting APLP2 may be facilitating the ability of cancer to metastasise (Pandey et al., 2015). The mechanism of action of APLP2 have been shown to involve APLP2 binding to MHC-I molecules at the cell surface resulting in the endocytosis of MHC-I. This reduction in cell surface MHC-I molecules reduces the presentation of cancer antigens and the surveillance and removal of cancer cells by CD8+ cytotoxic T cells (Peters et al., 2011; Tuli et al., 2009a; Tuli et al., 2008).

Such a mechanism is relevant to MND since MHC-I molecules are markedly reduced in motor neuron soma but increased in the motor axons in SOD1-G93A mice following disease onset. However, MHC-I is absent from either familial or sporadic MND spinal cords (Nardo et al., 2013; Song et al., 2016), where the MHC-I has transported away from motor neuron soma and accumulates in peripheral motor axons during disease progression (Chiarotto et al., 2017; Nardo et al., 2013). A high accumulation of MHC-I was observed in the NMJs of SOD1-G93A mice during disease progression (Chiarotto et al., 2017). These data suggest the activation of MHC-I in motor neurons of SOD1 mice at the onset of disease may be predictive of better preservation of axonal function, with a positive impact on disease progression.

Based on the association of APLP2 and MHC-I in cancer, it is possible that the upregulation of APLP2 in SOD1 transgenic may be detrimental, and its mechanism of action is via interacting with MHC-I and preventing axonal regeneration. Given that
APLP2 is known to associate with MHC-I molecule (Sester et al., 2000), the absence of APLP2 in the SOD1:APLP2/- mice may cause the MHC-I molecules to be upregulated at the NMJs as they cannot interact with APLP2. This, therefore, delays NMJ denervation, attenuates muscle atrophy, ameliorates motor function and extends survival of SOD1:APLP2/- mice as observed in our study.

Clearly, this model requires further investigation to establish the functional association between APLP2 and MHC-I in axonal regeneration and MND disease pathogenesis. One in vivo approach would be to conduct APLP2 and MHC-I expression studies during disease propagation in MND transgenic mouse model to test this possibility. Also, in vitro experiments using transfection of either APLP2 or MHC with green fluorescent protein (GFP) tagged in motor neurons differentiated from MND patient induced pluripotent stem cells (iPSCs) would allow us to further examine how APLP2 affects MHC-I transport in MND. For instance, the inhibition or over-expression of APLP2 in MND patient derived iPSCs would enable the investigation of APLP2’s impact on MHCs internalisation and recycling within the clathrin mediated endocytosis pathway (Adiko et al., 2015; Radhakrishna and Donaldson, 1997).

While questions regarding the overlapping but distinct, functional roles of APP and APLP2 in MND remain unanswered, it is clear that APP and APLP2 can modulate the pathophysiology of MND in a sex-dependent manner. The results of this study highlight the value of analysing both sexes in preclinical studies.

This thesis also highlights that APLP2 (and APP) represents a novel therapeutic target for the treatment and/or prevention of MND. Our findings also indicate that APLP2 plays a sex dimorphic role in MND and opens the prospect for a female specific intervention by targeting APLP2 that either inhibit its function and/or expression in female MND patients during disease progression. A better understanding of the sex specific functions of APLP2 (and APP) in MND may lead to the discovery of novel ways to modulate MND disease and contribute to the development of a more effective, gender responsive and personalised medicine.
Bibliography


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toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1. Science 281, 1851-1854.


Hilton, J.B., Kysenius, K., White, A.R., and Crouch, P.J. (2018). The accumulation of enzymatically inactive cuproenzymes is a CNS-specific phenomenon of the SOD1(G37R) mouse model of ALS and can be restored by overexpressing the human copper transporter hCTR1. Exp Neurol 307, 118-128.


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regulate the nuclear translocation of the intracellular C-terminal domain (ICD) of the APP family of proteins. Biochemistry 42, 6664-6673.


Appendices

Appendix A

Histology buffers and reagents

ATPase muscle stain

All stock buffer solutions were kept for no longer than three weeks.

**ATPase pH 4.3**

6.5 ml 1M HCl  
10 ml 1M sodium acetate  
Allow solution to equilibrate over night at room temperature.  
Adjust pH to pH 4.3. Bring volume to 100 ml with MilliQ water. Store at 4°C.

**ATPase pH 10.2**

This solution was made fresh before each use.  
19 mg adenosine 5'-triphosphate disodium salt (ATP)  
2.50 ml 0.1 M sodium barbiturate  
1.25 ml 0.18 M calcium chloride  
8.75 mL MilliQ water  
Adjust to pH 10.2 with 5N NaOH. Filter solution and warm to 37°C before use.

Immunohistochemistry

**Blocking buffer (10% goat serum in DPBS)**

10 mL goat serum  
90 ml DPBS  
Store 10 mL aliquots at -20°C.

**Blocking buffer (10% horse serum in DPBS)**

10 mL horse serum  
90 ml DPBS  
Store 10mL aliquots at -20°C.

**4% Paraformaldehyde in M-PBS**

10 g Paraformaldehyde  
125 mL MilliQ water  
125 mL 2X M-PBS  
Method: To a 250mL bottle, add the paraformaldehyde and water and heat to 60°C (maximum temperature) on a heating block for 20 mins. Add 6-7 drops of 1N NaOH to the solution until paraformaldehyde fully dissolves. Remove from the heating block, cool to room temperature and add 2X PBS.  
Check pH using a pH stick only and adjusted to pH 7.5 with 1N NaOH if required. Filter and store at 4°C.
Permeabilization buffer (0.3% w/v Triton X-100 in blocking buffer)
300 μL  Triton X-100
100 mL  Blocking buffer
Store 10mL aliquots at -20°C.

70% ethanol
700 mL  Ethanol
300 mL  MilliQ water

90% ethanol
900 mL  Ethanol
100 mL  MilliQ water

Western blotting buffer and reagents

Brain and spinal cord lysis Buffer
(50 mM Tris-HCl, 150 mM NaCl, 0.1% TritonX-100, pH 7.5)
5 mL  1M Tris-HCl (pH 7.5)
7.5 ml 1M NaCl
50 μl  TritonX-100
1 tablet  Protease inhibitor (Invitrogen)
37.45 mL  Milli-Q water.
Mix. Store at 4°C.

Muscle lysis Buffer
(50 mM Tris-HCl, 150 mM NaCl, 0.1% TritonX-100, 0.1% SDS, 10 mM EDTA, 1 mM dithiothreitol)
5.00 mL  1M Tris-HCl (pH 7.5)
7.50 ml  1M NaCl
250 μl  20% SDS solution
50 μl  TritonX-100
1 mL  0.5M EDTA
50 μl  1 M dithiothreitol
1 tablet  Protease inhibitor cocktail (Invitrogen)
36.15 mL  Milli-Q water.
Mix and store at 4°C.

Blocking buffer for membrane (5% milk in PBS)
1 g  skim milk powder
20 mL  PBS

Laemmli sample buffer (2X)
(0.16M Tris, 4% SDS, 20% glycerol, 0.04% bromophenol blue)
40 mg bromophenol blue
16 mL 1M Tris-HCl pH 6.8
20 mL 20% SDS solution
20 mL glycerol
44 mL Milli-Q water.
Mix and store at room temperature.

Laemmli sample buffer (4X)
(0.25M Tris, 6% SDS, 40% glycerol, 0.04% bromophenol blue)
20 mg bromophenol blue
12.5 mL 1M Tris-HCl pH 6.8
15 mL 20% SDS solution
20 mL glycerol
2.5mL Milli-Q water
Mix and store at room temperature.

Transfer Buffer
200 mL 10X transfer buffer
300 mL methanol diluted
1.5 L MilliQ water.
Mix and store at 4°C.

Tris-buffered Saline (TBS)
50 mL 20X TBS
950 mL MilliQ water
Mix and store at room temperature.

Tris-buffered Saline Tween (TBST)
50 mL 20X TBS
0.5 mL Tween-20
950 mL MilliQ water.
Mix and store at room temperature.

Stock solutions

Tris-HCl pH 6.8 (1M)
121.14 g C₄H₁₁NO₃ (Tris)
800 ml MilliQ water
Adjust the pH to 6.8. Bring volume to 1 L.

Tris-HCl pH 7.5 (1M)
121.14 g C₄H₁₁NO₃ (Tris)
800 ml MilliQ water
Adjust the pH to 7.5. Bring volume to 1 L.
**APPENDICES**

**NaCl (1M)**
58.44 g NaCl
1000 ml MilliQ water

**Ammonium Sulphide solution (1% v/v)**
10 mL 20% Ammonium Sulphide (Sigma CAS #: 12135-76-1)
190 mL MilliQ water.

**Baker’s formal calcium**
1.00 g CaCl$_2$
100 ml 40% paraformaldehyde
Store at 4°C

**Calcium chloride (0.18M)**
1.99 g CaCl$_2$
100 ml MilliQ water
Store at 4°C.

**Calcium chloride (1%)**
1.00 g CaCl$_2$
100 ml MilliQ water
Store at 4°C.

**Cobalt chloride (2%)**
2.00 g CoCl$_2$.6H$_2$O
100 ml MilliQ water
Store at 4°C.

**Sodium acetate (1M)**
8.20 g CH$_3$COONa$_2$
100 ml MilliQ water
Store at 4°C.

**Sodium barbiturate (0.1M)**
2.06 g C$_8$H$_11$O$_3$N$_2$Na
100 ml MilliQ water
Store at 4°C.

**Phosphate-buffered Saline buffer (20X, PBS)**
170 g NaCl
6.9 g NaH$_2$PO$_4$.2H$_2$O
21.4 g Na$_2$HPO$_4$ (or 5.4g Na$_2$HPO$_4$.2H$_2$O)
900 mL MilliQ water.
Adjust the pH to 7.2. Bring volume to 1 L.
Store at room temperature.
Phosphate-buffered Saline buffer (PBS)
50 mL 20X PBS
950 mL MilliQ water
Filter sterilise or autoclave. Store at room temperature.

Phosphate-buffered Saline Tween buffer (PBST)
50 mL 20X PBS
0.5 mL Tween-20
950 mL MilliQ water
Filter sterilise or autoclave. Store at room temperature.

Dulbecco’s Phosphate Buffered Saline (D-PBS)
100 mL 10X M-PBS
900 mL Milli-Q water.
Store at room temperature. The pH was adjusted to 7.5.

Dulbecco’s Phosphate Buffered Saline (10X, D-PBS)
2.0 g KCl
2.0 g KH$_2$PO$_4$
80.0 g NaCl
21.6 g Na$_2$HPO$_4$·7H$_2$O
900 mL Milli-Q water.
Adjust the pH to 6.8 - 7.0. Bring volume to 1 L with Milli-Q water. Store at room temperature.

Mouse tonicity Phosphate Buffered Saline (10X, M-PBS)
87.00 g NaCl
6.25 g NaH$_2$PO$_4$·2H$_2$O
22.75 g Na$_2$HPO$_4$ (or 28.5 g Na$_2$HPO$_4$·2H$_2$O)
900 mL Milli-Q water.
Adjust the pH to pH to 6.8 - 7.0. Bring volume to 1 L with Milli-Q water.
Store at room temperature.

Mouse tonicity Phosphate Buffered Saline (2X, M-PBS)
200 mL 10X M-PBS
800 mL Milli-Q water.
Adjust the pH to 7.5 and store at room temperature.

Tris-buffered Saline (20X, TBS)
121.1 g Tris
175.3 g NaCl
1 L MilliQ water.
Mix and adjust the pH to 7.5 with HCl. Filter sterilise and store at room temperature.
Transfer Buffer (10X)
30.3 g Tris
144 g Glycine
1 L MilliQ water.
Mix and adjust the pH to 8.5-8.7. Filter sterilise and store at room temperature.
## Appendix B

### Behavioural monitoring

**Animal monitoring sheet**

- **Ethics Application #:** 1413304.1
- **Strains:** SODxAPLP2KO
- **Primary contact person:**
- **Contact details:**
- **Emergency contact details (AH):**
- **Principal Investigator:**
- **Contact details (BH):**
- **Emergency contact details (AH):**

**Cage or animal number(s) covered by this sheet:**

**Agreed frequency of monitoring:**

**Intervention:** As per Intervention Criteria Sheet

### Mouse monitoring sheet

<table>
<thead>
<tr>
<th>Date</th>
<th>Time</th>
<th>Normal</th>
<th>Mouse Weight (g)</th>
<th>Weight change</th>
<th>Abnormal appearance (hair loss or greying, &amp; body posture)</th>
<th>Abnormal behaviour</th>
<th>Abnormal response to provocation, vocalisation</th>
<th>Abnormal activity levels</th>
<th>Not eating or drinking</th>
<th>Body condition score (refer to attached scoring chart)</th>
<th>Impaired mobility</th>
<th>Difficulty breathing</th>
<th>Faeces</th>
<th>Eyes and nose</th>
<th>Other signs (Add comment)</th>
<th>Comments/Procedures performed/Actions taken (include Fate)</th>
<th>Researcher</th>
</tr>
</thead>
<tbody>
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<td></td>
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</table>

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## Intervention Criteria Sheet

**Steps:**  
1. Determine the severity of abnormalities seen, using the Severity Table below.  
2. Contact the AFM or AWO for advice if appropriate or required.  
3. Take action in accordance with the Intervention Criteria below.

### Intervention Criteria

Criteria are based on the severity of signs, as classified by the table below:

<table>
<thead>
<tr>
<th>Observations (based on the Severity Table below)</th>
<th>Action required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (✔ on monitoring sheet)</td>
<td>None</td>
</tr>
<tr>
<td>1 or more “mild to moderate” signs (based on below)</td>
<td>Increase frequency of observations to daily (seek AFM/AWO advice where appropriate so that treatment and care can be given)</td>
</tr>
<tr>
<td>1 or more “severe” signs (based on below)</td>
<td>Kill by cervical dislocation. If in doubt, seek second opinion from AFM/AWO.</td>
</tr>
</tbody>
</table>

### Severity Table

<table>
<thead>
<tr>
<th></th>
<th>Mild to Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Appearance</strong></td>
<td>Slight to moderate piloerection with no dehydration (skin tenting); slight evidence of not grooming.</td>
<td>Persistent piloerection with clear evidence of dehydration (skin tenting), poor coat condition, not grooming, hunched body, abdominal distension.</td>
</tr>
<tr>
<td><strong>Weight changes</strong></td>
<td>Slight to moderate weight decrease (&lt;15% compared to the average weight of 3 previous body weights)</td>
<td>Significant weight decrease (&gt;15% compared to the average weight of 3 previous body weights)</td>
</tr>
<tr>
<td><strong>Behaviour, Activity &amp; Response to provocation</strong></td>
<td>Subdued but responsive, decreased interaction with peers.</td>
<td>Persistently unresponsive or minimally responsive to activity and provocation.</td>
</tr>
</tbody>
</table>
| **Body condition score** | Score between 2 to +2.  
*See monitoring sheet for scoring system.* | Score -2 to 1 |
| **Decreased appetite or drinking** | Slight loss of body condition; slight weight loss (<15%); not interested in food or water. | Obvious loss of body condition; obvious weight loss (>15%); not eating or drinking for more than 24h. |
| **Interference with mobility/movement/gait** | Slight physical interference to mobility; can still move around, and access food and water | Interference with mobility/gait impairs the animal’s ability to move around freely or limits its |
without problems; no signs of pain or distress.

access to food and water; signs of pain or distress noted; evidence of loss of coordination or limb paralysis; <2 secs performance on rotarod; unable to stand up within 15 secs of being laid on side.

<table>
<thead>
<tr>
<th>Breathing</th>
<th>Rapid and shallow</th>
<th>Rapid abdominal breathing or laboured irregular breathing and skin blue.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eyes and nose</td>
<td>Wetness or dull eyes, some nasal discharge/squinty eyes</td>
<td>Coagulated nasal discharge/ matted eyes</td>
</tr>
<tr>
<td>Faeces</td>
<td>Moist but formed faeces, loose faeces and soiled peri-anal area</td>
<td>Watery or no faeces in 24 hrs, blood evident in faeces</td>
</tr>
<tr>
<td>Vocalisation</td>
<td>No vocalisation</td>
<td>Vocalisation, suggestive of pain or distress</td>
</tr>
<tr>
<td>Other signs</td>
<td>Seek AFM/AWO advice re: appropriate action or euthanase if animal is in moderate or severe pain or distress</td>
<td></td>
</tr>
</tbody>
</table>
**Mouse body condition score**

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>The mouse is obese, and bones cannot be felt at all.</td>
<td>No action is required.</td>
</tr>
<tr>
<td>4</td>
<td>The mouse is well-fleshed, and bones are barely felt</td>
<td>No action is required.</td>
</tr>
<tr>
<td>3</td>
<td>The mouse is in optimal condition. Bones are palpable but not prominent.</td>
<td>No action is required.</td>
</tr>
<tr>
<td>+2</td>
<td>The mouse is becoming thin and bones are becoming prominent</td>
<td>Closely monitor for changes in body mass.</td>
</tr>
<tr>
<td>2</td>
<td>The mouse is becoming thin and bones are prominent</td>
<td>Increase frequency of observations to daily. Closely monitor for changes in body mass. Seek AFM/AWO advice if appropriate.</td>
</tr>
<tr>
<td>-2</td>
<td>The mouse is thin, fat is becoming depleted and bones are prominent</td>
<td>Euthanasia</td>
</tr>
<tr>
<td>1</td>
<td>Muscle wasting is advanced, fat deposits are gone, and bones are very prominent.</td>
<td>Euthanasia</td>
</tr>
</tbody>
</table>
Figure C. 1 Representative PCR gel image of SOD1-G37R transgenic and WT mice.

Genomic DNAs prepared from mouse tail samples were separated by DNA agarose gel electrophoresis. Mice positive for SOD1 transgene showed two bands; top band at ~324bp representing the endogenous SOD1 transgene and a lower band at ~236bp representing the transgene. Mice positive for SOD1-G37R transgene have been labelled as SOD1 and non-transgenic littermates have been labelled as WT. Negative and positive controls were water and genomic DNA from WT animal respectively. PC: positive control; NG: negative control; BP: base pairs.
Figure C. 2 Human SOD1 protein expression in SOD1-G37R mice and WT controls. Representative immunoblots showing selective immunoreactivity of human SOD1 protein (hSOD1) only in the spinal cord tissues of SOD1-G37R (SOD1) transgenic mice at end stage but not in the WT littermates control. A consistent amount of Gapdh expression is shown in the bottom lane.
Appendix D

Chapter 5- Relative levels of APLP2, BACE1 and NRG1 in the brain, spinal cord and muscles of SOD1:APLP2 mouse lines

Figure D. 1 Relative protein levels of APLP2, BACE1 and NRG1 in the brain, spinal cord and hindlimb muscles of SOD1:APLP2 mouse lines

Western blot analysis of (A) APLP2, (B) BACE-1, and (C) neuregulin-1 (NRG-1) protein expression levels in the brain, spinal cord, and muscles of SOD1:APLP2+/+, SOD1:APLP2+/ and SOD1:APLP2-/ mice at end-stage of disease and WT:APLP2+/+, WT:APLP2/- age-matched littermate controls. For each genotype, the relative intensity data of female and male mice were and normalised to WT:APLP2+/+. Data represent mean ± SEM, one-way ANOVA with Bonferroni’s posthoc test, ****p<0.0001, N=4-6.
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Author/s:
Truong, Phan Hong

Title:
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2019

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