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Targeting the Ubiquitin Proteasome System to Develop Novel Therapeutic Approaches for Spinal Muscular Atrophy

Rachael Anita Powis

A thesis submitted for the degree of Doctor of Philosophy

The University of Edinburgh

2015
Declaration

I declare that I have composed the following thesis based upon my own work, unless stated in the text, which has not been submitted for any other degree or professional qualification.

Rachael Anita Powis
Acknowledgements

I would like to extend thanks to all the people who I have worked with for the past three years for their generous contributions to the studies included in this thesis.

First and foremost, thanks to Professor Tom Gillingwater for being a fantastic PhD supervisor and giving me the opportunity to work in his lab on such interesting projects. I really appreciate the continuous support, feedback and opportunities he has given me. I am especially grateful for all the help and encouragement when experiments don’t work and enthusiasm for when they do.

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analysis. Thanks to Ines for help over the past three years and for being a constant presence during my time in the lab while others have come and gone.

Finally, thanks to my family and friends back home; especially my mum, dad and brother for their unconditional support. I dedicate this thesis to them.
Abstract

Spinal muscular atrophy (SMA) is a severe genetic neuromuscular disorder characterised by lower motor neuron degeneration and paralysis. Although it is a leading genetic cause of childhood death no approved treatment options currently exist. As SMA is caused by low levels of the survival motor neuron (SMN) protein the majority of therapeutic strategies under development are therefore aimed at trying to elevate SMN levels. However, a number of limitations with these approaches exist demonstrating a need for the investigation of SMN-independent therapeutics. Of these non-classical pathways, the ubiquitin proteasome system (UPS) is an exciting new area of SMA research. The UPS is a system which degrades unwanted or damaged proteins and alterations in the UPS (including reduced levels of ubiquitin-like modifier activating enzyme 1 [Uba1] and increased levels of ubiquitin carboxyl-terminal esterase L1 [Uchl1] and β-catenin) have been recently identified in the neuromuscular system of SMA mice, providing promising new targets for therapy development.

In this thesis I demonstrate that UPS perturbations are also present in other organ systems of severe ‘Taiwanese’ SMA mice and in other SMA models including intermediate Smn2B/− mice, zebrafish and patient derived iPSC motor neurons. Given the previously demonstrated improved neuromuscular phenotype in SMA mice treated with the β-catenin inhibitor quercetin I have been establishing whether other compounds with β-catenin inhibition offer similar or even better therapeutic options. Aspirin, indomethacin and iCRT-14 trials did not improve the SMA phenotype with likely off-target adverse effects meaning that quercetin remains the most tolerable β-catenin inhibitor in SMA mice to date. Another potential target of the UPS for SMA therapeutics is the deubiquitinating enzyme Uchl1, levels of which are increased in SMA. In this thesis I show that pharmacological inhibition of Uchl1 did not improve survival or motor performance in SMA mice and instead had a detrimental impact on the disease phenotype which could be explained by worsening SMA ubiquitin defects. Histological analysis revealed that there was no improvement in lower motor neuron count numbers, neuromuscular junction deficits or muscle fibre diameters. Mimicking the UPS phenotype in primary neuronal cells suggested that targeting
UPS perturbations observed in SMA that are upstream of Uchl1, particularly the loss of Uba1, may therefore offer a more effective therapeutic option. Finally, I therefore examined whether increasing Uba1 levels in SMA mice using gene therapy technology was able to improve the SMA phenotype. My initial studies indicate that delivery of AAV9-UBA1 to SMA mice may be beneficial as intraperitoneal injection of AAV9-UBA1 was found to increase the weight and improve motor performance of SMA mice. Intravenous delivery of AAV9-UBA1 was found to further improve expression levels and biodistribution of AAV9-UBA1 in the central nervous system as well as systemically in all body organs and tissues. Western blot and proteomic analysis revealed that AAV9-UBA1 gene therapy is also able to correct downstream UPS perturbations found in SMA as well as increase SMN levels. Together, these results suggest that AAV9-UBA1 gene therapy is an exciting novel therapeutic approach for SMA.
Lay Summary

Spinal muscular atrophy (SMA) is a common childhood motor neuron disease which causes wasting of muscles and death of spinal cord motor neurons. This causes paralysis and in the most severe cases death can be expected before the age of two years. Unfortunately no approved treatments are currently available. Recent breakthrough research has found that a pathway called the ‘ubiquitin proteasome system (UPS)’ which helps cells to remove damaged proteins is disrupted in SMA. In this thesis I have used different drugs and a technology called ‘gene therapy’ to target different parts of the UPS pathway to see if this improves the symptoms of a SMA mouse model. I found that three different drugs which inhibit a protein called β-catenin did not improve the weight, motor performance or survival of SMA mice. Additionally, a drug which inhibits the activity of an enzyme called Uchl1 did also not improve the symptoms of SMA mice. Analysis of tissue form SMA mice with Uchl1 inhibition did not show improvements in motor neuron numbers or muscle damage. Mimicking SMA in cells showed that increases in Uchl1 seen in SMA are actually due to reduced activity of a different enzyme called Uba1. Uba1 levels are reduced in SMA and it has previously been shown that this is responsible for many of the symptoms of SMA. I therefore tested whether increasing Uba1 levels using gene therapy would be beneficial in SMA mice. UBA1 gene therapy was successful at increasing UBA1 levels in a wide range of different tissues including spinal cord motor neurons. The weights and motor performance of SMA mice was also improved due to increased UBA1 levels indicating that UBA1 gene therapy is a new therapeutic strategy for SMA.
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<tr>
<td>2OMePS</td>
<td>2′-O-methyl phosphorothioate</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatosis polyposis coli</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis signal-regulating kinase 1</td>
</tr>
<tr>
<td>ASO</td>
<td>Antisense oligonucleotide</td>
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<tr>
<td>Atg8</td>
<td>Autophagy-related protein 8</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BME</td>
<td>Basal Medium Eagle</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CARM1</td>
<td>Coactivator-associated arginine methyltransferase 1</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CK1</td>
<td>Casein kinase 1</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COXIV</td>
<td>Cytochrome c oxidase subunit IV</td>
</tr>
<tr>
<td>Cpg15</td>
<td>Candidate plasticity-related gene 15</td>
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<tr>
<td>DABCO</td>
<td>1,4,-diazobicycl-[2.2.2]-octane</td>
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<td>DMSO</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>Nucleoside triphosphate</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DSBH</td>
<td>Developmental Studies Hybridoma Bank</td>
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<tr>
<td>DUB</td>
<td>Deubiquitinating enzymes</td>
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<td>Embryonic day</td>
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<td>E1</td>
<td>Ubiquitin activating enzyme</td>
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<td>Ubiquitin chain elongation enzyme</td>
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<td>EDTA</td>
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<td>FDA</td>
<td>US Food and Drug Administration</td>
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<td>FL-SMN</td>
<td>Full length survival motor neuron protein</td>
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<td>FMRP</td>
<td>Fragile X mental retardation protein</td>
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<tr>
<td>FTDP-17</td>
<td>Fronto-temporal dementia and parkinsonism linked to chromosome 17</td>
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<td>Fused in sarcoma</td>
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<td>GAPDH</td>
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<td>HDACi</td>
<td>Histone deacetylase inhibitor</td>
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<td>hiPSCs</td>
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<td>hnRNP</td>
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<td>HPRT</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
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<td>ICV</td>
<td>Intracerebroventricular</td>
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<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
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<td>IGF-binding protein acid labile subunit</td>
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<td>Immunoglobulin G</td>
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<td>Insulin-like growth factor 2 mRNA binding protein 1</td>
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<td>Ingenuity pathway analysis</td>
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<td>KSRP</td>
<td>KH-type splicing regulatory protein</td>
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<td>LC-MS</td>
<td>Liquid-chromatography mass-spectrometry</td>
</tr>
<tr>
<td>LRP6</td>
<td>Lipoprotein receptor-related protein 6</td>
</tr>
<tr>
<td>Lsm proteins</td>
<td>Sm like proteins</td>
</tr>
<tr>
<td>Mib1</td>
<td>Mindbomb</td>
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<tr>
<td>Min</td>
<td>Minutes</td>
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<tr>
<td>miR-183</td>
<td>MicroRNA 183</td>
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<tr>
<td>miRNA</td>
<td>MicroRNA</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------------------------------------------------------------------------</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NAIP</td>
<td>Neuronal apoptosis inhibitory protein gene</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NEDD8</td>
<td>Neural precursor cell-expressed developmentally downregulated-8</td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>N-Acetylneuraminic acid</td>
</tr>
<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
</tr>
<tr>
<td>NSCs</td>
<td>Neural stem cells</td>
</tr>
<tr>
<td>OAZ1</td>
<td>Ornithine decarboxylase antizyme 1</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature compound</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>P</td>
<td>Postnatal day</td>
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<tr>
<td>p38 MAPK</td>
<td>p38 mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PB</td>
<td>Sodium phenylbutyrate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pICln</td>
<td>Chloride conductance regulatory channel</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PPIA</td>
<td>Peptidyl-prolyl cis-trans isomerase</td>
</tr>
<tr>
<td>PRMT</td>
<td>Arginine methyltransferase</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RhoA</td>
<td>Rho factor A</td>
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<tr>
<td>RIPA buffer</td>
<td>Radioimmunoprecipitation assay buffer</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real-time reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SAHA</td>
<td>Suberoylanilide hydroxamic acid</td>
</tr>
<tr>
<td>ScAAV</td>
<td>Self-complementary adeno-associated virus</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<tr>
<td>Sec</td>
<td>Seconds</td>
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<tr>
<td>SERF</td>
<td>Small EDRK-Rich Factor gene</td>
</tr>
<tr>
<td>SF2/ASF</td>
<td>Serine arginine protein alternative splicing factor 1/ pre-mRNA-splicing factor</td>
</tr>
<tr>
<td>SK channel</td>
<td>Small conductance calcium-activated potassium channel</td>
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<tr>
<td>SMA</td>
<td>Spinal muscular atrophy</td>
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<tr>
<td>SMN</td>
<td>Human survival motor neuron protein</td>
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<tr>
<td>Smn</td>
<td>Non-human survival motor neuron gene</td>
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<tr>
<td>SMN1/SMN1</td>
<td>Human survival motor neuron genes</td>
</tr>
<tr>
<td>SMNΔ7</td>
<td>Delta7 survival motor neuron protein</td>
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<tr>
<td>snoRNP</td>
<td>Small nucleolar ribonucleoproteins</td>
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<tr>
<td>snRNA</td>
<td>Small nuclear RNA</td>
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<tr>
<td>snRNP</td>
<td>Spliceosomal small nuclear ribonucleoprotein</td>
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<tr>
<td>STAT5</td>
<td>Signal transducers and activators of transcription 5</td>
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<tr>
<td>SUMO</td>
<td>Small ubiquitin-related modifier</td>
</tr>
<tr>
<td>SV2</td>
<td>Synaptic vesicle protein 2</td>
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<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TDP-43</td>
<td>TAR DNA-binding protein 43</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
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<tr>
<td>THET</td>
<td>Snn heterozygote mouse line</td>
</tr>
<tr>
<td>THOM</td>
<td>Taiwanese homozygote mouse line</td>
</tr>
<tr>
<td>TRA2B / 2B</td>
<td>Transformer-2 protein homolog beta</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>TTG</td>
<td>Taiwanese heterozygote SMA mouse line</td>
</tr>
<tr>
<td>TVA</td>
<td>Transversus abdominis muscle</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UBA1</td>
<td>Ubiquitin-like modifier activating enzyme 1</td>
</tr>
<tr>
<td>UCHL1</td>
<td>Ubiquitin carboxyl-terminal hydrolase L1</td>
</tr>
<tr>
<td>Unrip</td>
<td>UNR-interacting protein</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin proteasome system</td>
</tr>
<tr>
<td>Usp9x</td>
<td>Ubiquitin specific peptidase 9, X-linked</td>
</tr>
<tr>
<td>Vg</td>
<td>Vector genomes</td>
</tr>
<tr>
<td>VPA</td>
<td>Valproic acid</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-type MMTV integration site1</td>
</tr>
<tr>
<td>YAC</td>
<td>Yeast artificial chromosome</td>
</tr>
<tr>
<td>YG box</td>
<td>Tyrosine-glycine rich domain of SMN protein</td>
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Chapter 1. Introduction

1.1 Spinal muscular atrophy: a clinical overview

Proximal spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder that, with an incidence of 1 in 6,000 to 10,000 live births, is a leading genetic cause of infant mortality (Pearn, 1978, Lunn and Wang, 2008). The hallmark clinical signs of lower motor neuron degeneration and proximal muscle atrophy which gradually progresses to trunk muscle involvement (Lunn and Wang, 2008). The resulting muscle weakness leads to a progressive paralysis, respiratory distress and ultimately death in the most severe cases (Lunn and Wang, 2008). Loss of neurons from the anterior horn of the spinal cord is often accompanied by reactive gliosis, chromatolysis and peripheral nerve degeneration (Kuru et al., 2009, Chien and Nonaka, 1989, Araki et al., 2003). Electromyography characteristics reveal evidence of denervation and spontaneous fasciculations (Buchthal and Olsen, 1970) whilst muscle histology shows that alongside atrophied muscle fibres a significant proportion of compensatory hypertrophic fibres are present (Kingma et al., 1991, Yiu et al., 2008, Millino et al., 2009). More recently, histological and electrophysiological neuromuscular junction (NMJ) abnormalities have also been identified in SMA patients (Kariya et al., 2008, Wadman et al., 2012). Alongside this classical neuromuscular phenotype, more recent evidence supports the fact that other organ systems are also affected (Hamilton and Gillingwater, 2013) although there is no overt decline in cognitive function or intellectual ability (Carter et al., 1995).

Clinically, SMA can be classified into four main classical types based on age onset and motor milestones ranging from the most severe Type I affecting infants under six months to the least severe adult onset, Type IV (Dubowitz, 1995, Zerres and Rudnik-Schoneborn, 1995, Pearn, 1980, Munsat and Davies, 1992).

Some groups also recognise a prenatal form of the disease called Type 0 (Dubowitz, 1999). The proposed clinical diagnostic Type 0 SMA has an in utero onset with a lifespan of 2 to 6 months after birth (Dubowitz, 1999). The signs and symptoms of this incredibly severe form include reduced foetal movement during pregnancy and
abnormal positioning of limbs and joint contractures visible on ultrasound scans (Macleod et al., 1999). There is negligible motor development with little or no detection of movement by the pregnant mother and severe weakness at birth (Dubowitz, 1999, Macleod et al., 1999).

Type I SMA (also known as Werdnig-Hoffmann disease after the two German neurologists Guido Werdnig and Johan Hoffmann who first accurately described the disorder in the early 1890’s (Werdnig, 1891, Hoffman, 1891)) is the most common type of SMA with around 60% of SMA sufferers presenting with this form of the disease. Diagnosis of Type I SMA usually occurs around 6 months of age with death usually occurring within the first 2 years of life (Werdnig, 1891, Hoffman, 1891). Type I SMA patients present with flaccid paralysis with only minimal muscle tone and are unable to perform head movements or sit unsupported (Lunn and Wang, 2008). This profound hypotonia sometimes leads to Type I SMA being referred to as “floppy baby syndrome”. The relative sparing of the diaphragm and intercostal muscles results in a ‘bell-shaped’ torso and paradoxical breathing where the abdomen moves out whilst the thorax swings inward (Lunn and Wang, 2008).

Tongue fasciculations are also present with upper respiratory tract weakness due to bulbar denervation resulting in increased risk of aspiration pneumonia (Lunn and Wang, 2008). Due to these breathing difficulties a significant number of parents of Type I children opt for artificial ventilation via tracheostomy. This procedure also increases the risk of respiratory infection, which is a major cause of mortality in these patients (Cobben et al., 2008). Problems swallowing effectively may require the patient to be fitted with a feeding tube for nutritional support (Wang et al., 2007).

Weakness can also lead to scoliosis and hip displacement, which can be treated with support from a brace or surgery in some instances (Haaker and Fujak, 2013). Problems may arise with other organ systems, including the heart (Distefano et al., 1994), vasculature (Rudnik-Schoneborn et al., 2010), lungs (Leistikow et al., 1999), pancreas (Bowerman et al., 2012b) and bones which are weak and can easily break (Aton et al., 2014, Shanmugarajan et al., 2007). The quality of life of Type I children is low, especially for those on long-term respiratory support (Gray et al., 2013). In some cases, physicians have argued that long-term ventilation it is not in the best
interests of Type I SMA children as the benefits of prolonged life do not outweigh the significant burdens of the disease (Gray et al., 2013, Wilkinson and Gillam, 2013). There are, however, a number of therapeutic supports, including ventilation and nutritional support as well as spinal surgery and wheelchairs to aid mobility, that can help children affected by SMA live more comfortably (Wang et al., 2007).

Type II is an intermediate type of SMA (also referred to as Dubowitz disease after the neurologist Victor Dubowitz who helped pioneer the classification system for SMA (Dubowitz, 1995)) with an age of onset between 7 and 18 months and diagnosis usually made before 2 years of age. Death occurs during adolescence with approximately 70% of patients surviving to 25 years of age (Lunn and Wang, 2008). The severity is less than Type I SMA, with Type II patients being able to sit independently and although some may stand with support none can walk unaided (Lunn and Wang, 2008). Tremors of the hand digits are common as well as muscle twitching in the limbs and/or tongue and deformity of joints (Lunn and Wang, 2008). Electrocardiogram abnormalities can also be present at a high incidence (Huang et al., 1996). Vertebral problems usually develop with many children undergoing surgery to correct kyphoscoliosis (Granata et al., 1993). Bulbar denervation is also present in Type II patients; with weakness in swallowing muscles often preventing weight gain during development and respiratory insufficiency also leading to morbidity and death as with Type I children (Lunn and Wang, 2008).

The mild Type III SMA is also called juvenile SMA or Kugelberg-Welander disease (named after Erik Kugelberg and Lisa Welander after their description of a more slowly progressing SMA and distinguishing it from muscular dystrophy (Kugelberg and Welander, 1956)). This form of the disease has an onset occurring after 18 months of life and is typically diagnosed within 3 years of birth although later diagnosis in teenage years can occur (Lunn and Wang, 2008). All patients in this group reach major motor milestones including independent standing and walking although some patients may lose this ability with age (Lunn and Wang, 2008). The clinical phenotype of Type III SMA can vary widely; some patients may require wheelchair assistance in childhood whilst some display only mild muscle weakness.
(Lunn and Wang, 2008). This hypotonia can lead to overuse of joints and scoliosis is a common occurrence (Granata et al., 1993). Despite this, Type III SMA patients often live until adulthood with a normal lifespan, however postmortem examination reveals anterior horn neuronal loss, marked neurogenic change of skeletal muscle and cardiomyocyte degeneration (Kuru et al., 2009).

Type IV is very mild adult form of SMA with symptom onset not until the patient is in their twenties or thirties (Lunn and Wang, 2008). As with Type III all milestones are achieved including the ability to walk and only a mild motor impairment of muscle weakness occurs (Lunn and Wang, 2008). As no major respiratory or nutritional problems are present a full lifespan can be expected (Munsat and Davies, 1992).

Although this classification system is useful, SMA is extremely heterogeneous and patients may not fall cleanly in to one category (Dubowitz, 1995). SMA can be also often misdiagnosed due to similarities with other genetically distinct neurological and muscular disorders. Numerous rarer spinal muscular atrophies exist with different underlying genetic causes. Some of the most common of these disorders include the X-linked spinal and bulbar muscular atrophy (also known as Kennedy’s disease) (La Spada et al., 1991), spinal muscular atrophy with respiratory distress (Grohmann et al., 2001) and congenital distal spinal muscular atrophy (Auer-Grumbach et al., 2010).

### 1.2 The genetics of SMA

SMA is caused by low levels of the survival motor neuron (SMN) protein (Lefebvre et al., 1995). The gene encoding the SMN protein, survival motor neuron (SMN), was first identified as the SMA causing determinant in 1995 (Lefebvre et al., 1995). Mapping the SMA locus by conventional methods was complicated due to the clinical heterogeneity, the rapidly fatal course and its recessive nature (Gilliam et al., 1990). In 1990, two independent linkage analysis studies of clinically heterogeneous Type I-III SMA families were published simultaneously, which found that chronic
childhood onset SMA mapped to a single locus on the long arm of chromosome 5 (Brzustowicz et al., 1990, Melki et al., 1990). Physical mapping of this locus revealed that the putative SMA disease containing genomic region was highly unstable including the presence of pseudogenes and complex repetitive gene sequence arrangements (Melki et al., 1994, Carpten et al., 1994, Theodosiou et al., 1994). Analysis of allelic segregation in 201 SMA families also first identified that deletion events were statistically associated with SMA (Melki et al., 1994). The physical organization of SMN containing region was ultimately elucidated by long-range restriction mapping of yeast artificial chromosome (YAC) contigs containing polymorphic loci previously detected by microsatellite markers (Lefebvre et al., 1995). Phage libraries of YACs were created and subclones of phages containing promising microsatellites used as probes for pulse field gel electrophoresis analysis and locations of the markers determined by PCR screening (Lefebvre et al., 1995).

This analysis by Lefebvre et al. (1995) revealed that in humans the SMN protein is encoded by two genes denoted survival motor neuron 1 gene (SMN1) and its duplicate survival motor neuron 2 (SMN2). These SMN gene copies are found in two large inverted chromosome fragments on chromosome 5q13.3 (with SMN1 the telomeric copy and SMN2 the centromeric copy) arranged in a head-to-head orientation (Figure 1-1A) (Lefebvre et al., 1995). Both genes span ~20kb and consist of 9 exons and 8 introns and are regulated by near identical promoter elements (Lefebvre et al., 1995, Burglen et al., 1996). Sequencing revealed that the two genes were identical apart from 5 nucleotide substitutions: 3 present in introns and 1 present in both exon 7 and exon 8 (Figure 1-1B) (Lefebvre et al., 1995, Burglen et al., 1996). The presence of the SMN2 gene in humans represents a unique genetic divergence whereby in the majority of metazoan organisms the SMN gene orthologue is present in a single copy and although primates possess an evolutionary duplication of the Smn gene no SMN2 equivalent is found (Rochette et al., 2001). Low levels of SMN in SMA arise from homozygous disruption (in the form of deletions [loss of exon 7 or exon 7 and 8 in 95% of cases], rearrangements or mutations [Y272C being the most common missense mutation] (Wirth, 2000)) in SMN1 which renders the gene non-functional and unable to encode SMN protein (Lefebvre et al., 1995). Loss
SMN2 harbours an important base pair difference in the last coding exon (Lefebvre et al., 1995). A transitionally silent C-to-T nucleotide variation at position 6 in exon 7 means that the resulting pre-mRNA contains a G-to-U mutation which is responsible to the skipping of exon 7 in 80-90% of transcripts (Figure 1-1D) (Lorson et al., 1999, Monani et al., 1999). This dramatic effect on SMN2 mRNA processing is due to the exon 7 C-to-T point mutation both disrupting an exon splice enhancer site that binds the serine arginine (SR) protein alternative splicing factor 1/ pre-mRNA-splicing factor (SF2/ASF) (Cartegni and Krainer, 2002) and/or generating a new exonic splice silencer that binds heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) (Kashima and Manley, 2003, Kashima et al., 2007b, Kashima et al., 2007a). These alternatively spliced SMN2 mRNA transcripts which lack exon 7 produce a rapidly degraded truncated version of the SMN protein called SMNΔ7 (Figure 1-1D) (Lorson et al., 1999, Monani et al., 1999). SMNΔ7 lacks the SMN C-terminus residue and due to the generation of a new stop codon in SMNΔ7 mRNA is replaced by 4 amino acids encoded by exon 8 (Lefebvre et al., 1995). Both full-length SMN (FL-SMN) and SMNΔ7 mRNA have a similar half-life indicating equivalent stability of both SMN2 transcript products (Heier et al., 2007).

The instability and reduced activity of SMNΔ7 protein is thought to be due the SMN protein being unable to oligomerise and form complexes with its usual protein and RNA binding partners due to an altered C-terminus (Lorson et al., 1998, Pellizzoni et al., 1999, Vitte et al., 2007, Burnett et al., 2009). Roughly 10% of the SMN2 mRNA transcripts, however, do undergo correct splicing, producing a small amount of functional FL-SMN protein (Lorson et al., 1999). As complete absence of Smn is not compatible with life (Schrank et al., 1997), the SMN2 gene ensures that loss of SMN1
is embryonically viable in humans but is unable to compensate fully, resulting in the
SMA phenotype (Lefebvre et al., 1995). The chromosomal region harbouring the
SMN genes is inherently unstable resulting in the potential presence of multiple
SMN2 copies in humans (Mcandrew et al., 1997). The greater the number of SMN2
copies, the more FL-SMN protein is ultimately translated and as SMA disease
severity is dependent on the amount of functional SMN protein (Lefebvre et al.,
1997), severity is therefore inversely correlated to SMN2 copy number with Type I
patients generally possessing one or two SMN2 copies and Type IV having four to
six SMN2 copies (Feldkotter et al., 2002, Wirth et al., 2006). However, this
relationship between SMN2 copy number and symptom severity doesn’t fully hold in
all cases, with patients having an identical number of SMN2 copies but different
phenotypes being reported (Wang et al., 1996, Zheleznyakova et al., 2011, Harding
et al., 2015). For example, a c.859G>C substitution in exon 7 of SMN2 (which
creates a new exonic splicing enhancer element and increases the amount of FL-
SMN transcripts) was found to be a positive modifier of SMA with patients
harbouring this substitution having a milder clinical phenotype and is particularly
associated with SMA Types II and III rather than Type I (Prior et al., 2009, Bernal et
al., 2010).
Figure 1-1. Schematic of the SMN1 and SMN2 genes and SMN protein. A. Genetic map of human chromosome 5q13 containing the SMN1 and SMN2 genes. B. Nucleotide differences between the SMN genes with SMN1 sequences on top and SMN2 on bottom, including the C-to-T transition in exon7 important in SMN2 splicing. C. The SMN protein and its subdomains. D. Overview of differential SMN1 and SMN2 mRNA processing and immunohistochemistry showing normal and SMN deficient motor neurons. Adapted from (Sendtner, 2010, Lunn and Wang, 2008, Coady and Lorson, 2011).
1.3 The SMN protein

The SMN protein consists of 294 amino acids with a molecular weight of 38 kDa (Lefebvre et al., 1995). SMN is a multidomain polypeptide with an N-terminal lysine (K)-rich sequence, a central Tudor domain and C-terminal containing a proline (P)-rich sequence and a tyrosine-glycine (YG)-box all serving different functional properties (Figure 1-1C) (Coady and Lorson, 2011). The K-rich sequence encoded by exon 2a and 2b harbours a nucleic acid binding domain, with 2a encoding the majority of the gemin-2 binding region and 2b a critical region for self-association and Cajal body targeting (Lorson and Androphy, 1998, Young et al., 2000, Morse et al., 2007). Exon 3 encodes the Tudor domain (a conserved structural motif present in many RNA binding proteins so called after its original identification in the Drosophila Tudor protein) which facilitates the direct binding of Sm proteins with SMA causing point mutations in this region preventing SMN-Sm protein interaction (Buhler et al., 1999, Selenko et al., 2001, Sprangers et al., 2003). The P-rich sequence encoded by exon 5 mediates binding of profilin, an actin binding partner, with altered profilin expression following SMN knockdown in cell culture affecting cytoskeletal integrity and causing defects in neuritogenesis (Giesemann et al., 1999, Bowerman et al., 2007). The YG-box of exon 6 is required for self-association with oligomerisation essential for SMN protein function (Lorson et al., 1998, Talbot et al., 1997). Missense mutations in this region can be found in SMA patients with oligomerisation defects correlated to SMA severity (Lorson et al., 1998, Talbot et al., 1997). As seen from investigations into the SMNΔ7 protein, exon 7 encodes a region important for stability (Burnett et al., 2009). Exon 7 is also necessary for the localization of SMN in to the cytoplasm and the cytoskeletal-based active transport of SMN in neuronal processes (Zhang et al., 2003, Hua and Zhou, 2004a, Zhang et al., 2007, Frugier et al., 2000).

SMN is evolutionary conserved including orthologues present in the Fungi kingdom (Paushkin et al., 2000). It is ubiquitously expressed with particularly high levels in spinal motor neurons (Battaglia et al., 1997) which is reflected in a marked reduction of SMN in lower motor neurons compared to other cell types in SMA patients.
(Coovert et al., 1997). Expression of the SMN protein is highly regulated during development with high levels in the embryo and foetus which decline to relatively low levels in adult tissues (Burlet et al., 1998, La Bella et al., 1998). Within the cell, SMN is present both in the nucleus and cytoplasm, localizing in the former into small foci called ‘gems’ (short for Gemini of coiled/Cajal bodies) (Liu and Dreyfuss, 1996). As the name suggests, gems are found in close proximity to Cajal bodies, although the precise function of gems is still unknown but are thought to serve as sites of RNA processing (Pellizzoni et al., 1998). SMA patients have fewer gem numbers with gem counts being utilized as a useful readout of SMN levels (Coovert et al., 1997).

As reviewed by the following publications, SMN is thought to play a role in numerous cell processes partly due its large number of protein interactions (Meister et al., 2002, Paushkin et al., 2002). The most well characterised role of SMN is its function in spliceosomal small nuclear ribonucleoprotein (snRNP) assembly and pre-mRNA splicing (Fallini et al., 2011, Pellizzoni et al., 2002b). snRNPs act as part of the spliceosome to remove introns from pre-RNA to form mature RNA as part of RNA’s post-translational modification in the nucleus of eukaryotic cells (Wahl et al., 2009). snRNPs are composed of small nuclear RNA (snRNA) of approximately 150 nucleotides (also referred to as U-RNA due to a high uridine content) and a set of specific RNA binding proteins called Sm proteins. SMN functions in the cytoplasm to attach the snRNA with a specific set of Sm proteins, named B/B’, D1, D2F D3, E, F, and G, to form an active snRNP. There are five well characterized snRNPs: U1, U2, U4, U5 and U6 that make up the major spliceosome along with over 150 different proteins to participate in pre-mRNA intron removal (Matera and Wang, 2014). Briefly, during the intron removal process U1 binds to the 5’ intron splice site to initiate splicing (Mccullough and Berget, 2000) whilst U2 binds to the 3’ intron splice site (Douglas and Wood, 2011). U1 and U2 snRNPs subsequently interact to form the pre-spliceosome bringing the two intron splice sites within close proximity (Douglas and Wood, 2011). The U4/U5/U6 snRNP tricomplex then associates to form a complete spliceosome (Grainger and Beggs, 2005). Rearrangement causes U1 and U4 to be removed, leaving behind ‘complex C’ of the spliceosome (composed of
U2, U5 and U6) which possesses the catalytic activity required for splicing (Douglas and Wood, 2011). Following two transesterification reactions, the intron lariat is removed and the two exons ligated to form mature mRNA (Lodish H, 2000). Although this major spliceosome pathway removes the vast majority of introns (known as U2-type) in eukaryotes another minor spliceosome pathway also exists (Turunen et al., 2013). Composed of a group of less abundant snRNPs U11, U12, U4atac and U6atac, together with U5 which is shared with the major pathway, the minor spliceosome removes a rarer type of introns, known as U12-type, with atypical splice sites (Turunen et al., 2013). Removal of U12-type introns is slower than U2-type introns and unlike the major spliceosome, the minor variety is found in the nucleus rather than the cytoplasm (Turunen et al., 2013). Genes containing U12-type introns tend to encode proteins with ‘information processing functions’ including DNA repair/replication and RNA processing but also those relating to the cytoskeleton, vesicular transport and voltage gated ion channels (Turunen et al., 2013, Burge et al., 1998). Interestingly, it is this minor form thought to be most affected in SMA, where reduced levels of the minor snRNPs (Zhang et al., 2008, Gabanella et al., 2007, Lotti et al., 2012) and inhibition of some U12-type intron splicing have been reported (Lotti et al., 2012).

The role of SMN in snRNP assembly has been intricately described (Figure 1-2). In this role, SMN functions as part of a protein structure called the SMN complex, where SMN binds to a group of proteins called gemins. Gemins-2, -3, -5, -7 and -8 bind SMN directly whilst gemin-4 and -6 associate to SMN indirectly via binding with gemin-3 and -7, respectively (Liu et al., 1997, Charroux et al., 2000, Meister et al., 2001a, Gubitz et al., 2002, Pellizzoni et al., 2002a, Baccon et al., 2002, Carissimi et al., 2006). UNR-interacting protein (Unrip), a factor implicated in cap-independent translation also associates with the SMN complex to influence its cytosolic localisation (Grimmler et al., 2005, Meister et al., 2001a). A variety of protein substrates associate with the SMN complex which are known to play diverse roles in RNA processing including the previously mentioned Sm and Sm-like (Lsm) proteins, which are essential for RNA splicing (Liu et al., 1997, Battle et al., 2006a). Before snRNP assembly the 7 Sm proteins are bound to the chloride conductance regulatory
channel (pICln) which inhibits the spontaneous assembly of Sm proteins onto snRNA (Meister et al., 2001b) and SMN binding (Pu et al., 1999). Methylation of Sm proteins by arginine methyltransferase-5 (PRMT5) and -7 (PRMT7) occurs allowing the Sm proteins to be transferred to the SMN complex (Meister et al., 2001b, Gonsalvez et al., 2007). The translated snRNAs are exported to and processed in the cytoplasm to form a 3’ stem loop structure which is necessary for recognition by the SMN complex and a 5’ 7-methylguanosine cap required for the eventual return of the completed snRNP back to the nucleus (Golembeski et al., 2005, Jacobson and Pederson, 1998). The snRNAs are identified and bound by geminin5 which brings the snRNA into the SMN complex (Battle et al., 2006b). Here, Sm proteins assemble onto a short, highly conserved, uridine rich sequence of the snRNAs known as the Sm site in a heptameric ring formation (Stark et al., 2001). In the assembled snRNPs, the snRNA 5’-cap is now hypermethylated which, along with the Sm ring formation, signals for snurportin-1 (a snRNP specific nuclear import adapter) to transfer the snRNP-bound SMN complex to be transported from the cytoplasm to the nucleus via the nuclear transport receptor importin-β (Fischer and Luhrmann, 1990, Plessel et al., 1994, Huber et al., 1998, Palacios et al., 1997). Here the snRNPs accumulate in Cajal bodies and can undergo further maturation and formation of higher order complexes before joining the spliceosome (Carvalho et al., 1999). Low levels of SMN reduce snRNP assembly activity, a defect that is correlated with SMA disease severity (Gabanella et al., 2007). Depletion of SMN also induces defects in Cajal body formation (Girard et al., 2006).

The SMN protein can also interact with the small nucleolar ribonucleoproteins (snoRNPs) fibrillarin and GAR1 which help guide site specific cleavage and methylation (C/D box snoRNAs) or pseudouridylation (H/ACA box snoRNAs) of ribosomal RNA respectfully (Pellizzoni et al., 2001). The capacity of interaction between SMN and the snoRNPs fibrillarin and GAR1 is reduced in SMNΔ7 protein and by Y272C SMN1 mutations found in patients indicating that snoRNP function may be defective in SMA (Pellizzoni et al., 2001). Additionally SMN can also influence telomerase reconstitution by associating with the ribonucleoprotein
telomerase (Bachand et al., 2002) suggesting that functions of the SMN complex can influence a variety of ribonucleoproteins and is not just limited to snRNP assembly.

Alongside its canonical role in ribonucleoprotein function, more recent evidence suggests additional roles of SMN, particularly in local post-transcriptional regulation (Fallini et al., 2012, Li et al., 2014). SMN has been shown to bind many RNA-binding proteins, including: HuD (Akten et al., 2011, Hubers et al., 2011, Fallini et al., 2011), KH-type splicing regulatory protein (KSRP) (Tadesse et al., 2008), insulin-like growth factor 2 mRNA binding protein 1 (IMP1) (Fallini et al., 2014), fragile X mental retardation protein 2 (FMRP) (Piazzon et al., 2008), heterogeneous nuclear ribonucleoproteins (hnRNP) (Rossoll et al., 2002, Mourelatos et al., 2001, Dombert et al., 2014), fused in sarcoma (FUS) (Yamazaki et al., 2012, Groen et al., 2013) and TAR DNA-binding protein 43 (TDP-43) (Bose et al., 2008). RNA-binding proteins have diverse functions within RNA biology including in transcription, premRNA splicing and polyadenylation, RNA modification, transport, localization, translation and turnover (reviewed by (Glisovic et al., 2008)). In primary neuronal culture, RNA-binding proteins are found in association with SMN (but not Sm proteins) in discrete RNA granules that shuttle along the axon bidirectionally (Akten et al., 2011, Hubers et al., 2011, Fallini et al., 2014, Fallini et al., 2011). This SMN binding facilitates RNA-binding protein trafficking into axons and regulates the localization and translation of mRNA (Akten et al., 2011, Sanchez et al., 2013, Rossoll et al., 2003). One of the most studied proteins subject to this SMN controlled regional localization is the cytoskeletal protein β-actin (Rossoll et al., 2003). Accordingly, primary motor neurons isolated from SMA mouse models are associated with a decreased neurite length and smaller growth cones (Rossoll et al., 2003). SMA associated SMN\(^1\) missense mutations can impair RNA-binding protein interaction and SMN deficiency also leads to reduction in RNA-binding protein levels and impaired mRNA axonal transport (Rossoll et al., 2003, Fallini et al., 2011).

Excitingly there is increasing evidence to suggest that SMN has additional functions outside of its well defined roles in RNA processing and transport. A novel finding

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that SMN can also associate with polyribosomes and act as a translational regulator was identified by Sanchez et al. (2013). In an *in vitro* assay SMN was able to repress translation, a function that is abolished with disease associated *SMN1* mutations (Sanchez et al., 2013). In particular, the translation of the arginine methyltransferase CARM1 was found to be increased in SMNΔ7 cells without alterations in transcription, mRNA stability or protein turnover (Sanchez et al., 2013). This increase in CARM1 levels was similarly identified in motor neurons of SMA mice and primary Type I patient fibroblasts indicating altered translational regulation of proteins could be contributing to disease pathogenesis (Sanchez et al., 2013). There is also an identified role of SMN in cell stress pathways (Hua and Zhou, 2004b, Zou et al., 2011). When overexpressed, SMN precedes accumulation of and colocalises with protein assemblers of stress granules indicating that SMN serves as a facilitator (Hua and Zhou, 2004b). SMN knockdown in cell culture reduces the ability of stress granule formation, thereby, sensitizing cells to stress and illustrating that SMN also plays an important role in cell survival (Zou et al., 2011).
Figure 1-2. The role of SMN in snRNP assembly. Following transcription of snRNA by RNA polymerase II, snRNA is exported from the nucleus where it partakes in snRNP assembly with Sm proteins. The SMN complex facilitates binding of methylated Sm proteins to the Sm site of SnRNA. Hypermethylation of the 5′SnRNA cap and binding of Sm proteins acts as a signal for the SnRNP bound-SNP complex to be imported to the nucleus via importin-β. SnRNPs localise to Cajal bodies where they undergo further maturation whilst the SMN complex localises to nearby gems. Figure adapted from (Paushkin et al., 2002).
1.4 Animal models of SMA

Although the genetic cause of SMA is well established the downstream molecular mechanisms of why low SMN protein levels cause the distinctive SMA phenotype are not fully understood. Since the identification of the SMA causing gene in 1995, considerable effort has been focused on generating animal models of SMA to further understand disease pathogenesis (Edens et al., 2014).

1.4.1 Drosophila melanogaster

The *Drosophila* orthologue of SMN gene (denoted *Smn*) shares 41% sequence homology with human SMN1 and encodes a SMN protein orthologue called dSMN (Miguel-Aliaga et al., 2000). Ectopic expression of human SMN1 or the C-terminal domain of Smn in *Drosophila* results in pupal lethality indicating that dSMN is essential for *Drosophila* survival and development (Miguel-Aliaga et al., 2000). *Drosophila* SMA models have been created based on various genetic mutations found in patients (Praveen et al., 2014). In keeping with the observed clinical variable severity, these *Drosophila* SMA models show a wide range of phenotypes with mutations which map to the YG box domain, which is important for SMN oligomerisation, resulting in a stronger phenotype (Praveen et al., 2014). In an independent study, a *Drosophila* model with YG box mutations showed late larval death and neuromuscular defects, including progressive loss of mobility, uncoordinated movement, reduced post-synaptic currents, disorganized motor neuron synaptic boutons and loss of glutamatergic receptor clusters at the NMJ (Chan et al., 2003). Rescue of *Smn* was required both in neurons and muscle to alleviate this phenotype (Chan et al., 2003). Hypomorphic mutants with a reduction of dSMN protein activity in the adult thorax show skeletal muscle atrophy, defects in motor neuron arborisation and are unable to fly (Rajendra et al., 2007). Mutants with larval lethality show no reduction in U2-type snRNA levels indicating another reason for death than dSMN related major-class snRNP deprivation (Rajendra et al., 2007). On the other hand, U12-type minor-class snRNAs were found to be decreased, however, this did not lead to defects in splicing of most mRNAs containing minor class introns (Praveen et al., 2012). One of the U12 intron-containing genes with reduced
expression in SMA Drosophila larve encodes the protein Stasimon, which has been shown to be important for motor function in flies (Lotti et al., 2012). Transgenic expression of human SMN is able to rescue lethality and motor defects of Smn null flies but is unable to restore snRNA levels, demonstrating that SMN’s snRNP biogenesis role may be uncoupled from its disease phenotype (Praveen et al., 2012). Indeed, differences in expression of minor intron containing genes may be explained as a system response to developmental arrest caused by low dSMN levels (Garcia et al., 2013).

Studies using Drosophila have also revealed roles for dSMN in varied biological processes, including: sensory-motor circuit function (Imlach et al., 2012), NMJ glutamate receptor-dependent developmental homeostasis (Timmerman and Sanyal, 2012), fibroblast growth factor signalling (Sen et al., 2011), stress responses (Garcia et al., 2013), apoptosis (Ilangoovan et al., 2003), germline nuclear organisation (Lee et al., 2009) and regulation of stem cell division, proliferation and differentiation (Grice and Liu, 2011). Drosophila are also useful as large-scale screening platforms and Drosophila models of SMA may be a useful tool for identifying SMN and phenotype modifying compounds. However, it is important to note that whilst mammalian neuromuscular synapses are cholinergic and sensory-motor synapses are glutamatergic; in flies the opposite is true (Imlach et al., 2012). Indeed SMN restoration to cholinergic proprioceptive neurons and interneurons was sufficient to rescue the phenotype of Smn null flies whereas SMN restoration to its glutamatergic motor system had no effect (Imlach et al., 2012). The previously mentioned Stasimon protein was also found to be required in cholinergic sensory neurons rather than motor neurons to rescue synaptic dysfunction and the phenotype of Drosophila Smn mutants (Lotti et al., 2012), illustrating that the comparison between fly and mammalian circuits is limited.

1.4.2 Caenorhabditis elegans

The nematode C.elegans ’ genome contains a SMN orthologue, Smn-1, that encodes a protein with 36% sequence homology to human SMN with conserved RNA binding properties (Bertrand et al., 1999). Knock-down of Smn-1 expression by RNA
interference is lethal but due to maternal transmission larvae are viable to late embryonic stages (Bertrandy et al., 1999). Disruption of CeSMN protein leads to growth arrest, locomotor dysfunction and defects in germ cell maturation which causes sterility (Briese et al., 2009). Neuronal rescue of Smn-1 in Smn-1 mutants leads to partial correction of this phenotype but muscle specific rescue does not, indicating that in C.elegans CeSMN functions primarily in neurons (Briese et al., 2009). Genetic screens have been conducted in C.elegans to find novel CeSMN binding partners (Burt et al., 2006) and to identify genes that could act to modify CeSMN loss of function defects (Dimitriadi et al., 2010). One class of such genetic modifiers identified was the small conductance calcium-activated potassium (SK) channel (Dimitriadi et al., 2013). Activating the SK channel with riluzole (a neuroprotective drug used in amyotrophic lateral sclerosis (ALS) therapeutics to improve survival by a modest extent) in a C.elegans SMA model was capable of restoring axon outgrowth defects and neuromuscular function (Dimitriadi et al., 2013). In a similar method to Drosophila, C.elegans also make a convenient drug-screening platform (Sleigh et al., 2011). A recent model with a point mutation resembling a less severe Type III SMA patient enabled the study of lack of CeSMN in adulthood with nematodes displaying impaired fertility, reduced locomotor ability and defective neurotransmission at the NMJ (Sleigh et al., 2011). A large-scale drug screen identified both US Food and Drug Administration (FDA) pre-approved and novel compounds that partially rescued the mutant phenotype (Sleigh et al., 2011). The most effective were 4-AP (a potassium channel blocker), gaboxadol (a GABA receptor antagonist) and Neu5Ac (a sialic acid commonly found in glycoproteins) suggesting that these compounds warrant further testing in vertebrate models of SMA (Sleigh et al., 2011).

1.4.3 Danio rerio

The zebrafish, D.rerio, Smn gene shares 52% sequence identity with human SMN1 (Bertrandy et al., 1999). As with the Drosophia orthologue, it encodes a Smn protein with conserved RNA binding properties in which SMA-like mutations interrupt the formation of RNA-protein binding complexes (Bertrandy et al., 1999). Reduction of Smn protein levels in zebrafish embryos using antisense morpholinos causes spinal
motor axon defects including impaired outgrowth and aberrant branching that preceded apoptosis, whereas muscle development, interneurons and sensory neurons remained unaffected (Mcwhorter et al., 2003). Introduction of human SMN1 mRNA was able to partially rescue this phenotype whereas SMNΔ7 mRNA could not (Mcwhorter et al., 2003). This axogenesis defect phenotype is remarkably similar to that seen in Xenopus SMA models (Winkler et al., 2005, Ymlahi-Ouazzani et al., 2010), however, such defects in axogenesis are not present in mouse models of SMA or human patients (Mcgovern et al., 2008, Murray et al., 2010b) indicating that the mechanisms which regulate correct neuromuscular synapse formation are different in lower vertebrates.

Motor neuron specific morpholino treatment to reduce Smn levels recapitulated the phenotype seen with knockdown in the entire embryo suggesting that Smn acts cell autonomously (Mcwhorter et al., 2003). However, recent studies from mouse models show that this is not the case in the mammalian system with motor neuron cell-nonautonomous SMN restoration able to rescue SMA phenotypes (Hua et al., 2015). Zebrafish with exon 7 truncation mutations or a missense mutation that corresponds to human SMA patients resulted in unstable Smn protein products (Boon et al., 2009). Homozygous mutants were able to live until the second week of development due to maternal Smn when they developed alterations at the NMJ particularly loss of the synaptic vesicle protein 2 (SV2) (Boon et al., 2009). Transgenic zebrafish that expressed human SMN driven by the hb9 motor neuron promoter were rescued of the SV2 NMJ defect indicating a requirement for Smn in motor neurons in normal presynaptic integrity (Boon et al., 2009). In order to create a model which more closely resembled SMA patients a transgenic zebrafish which expressed the human SMN2 gene on a smn null background was created (Hao Le et al., 2011). The presence of SMN2 was able to improve survival and correct the SV2 NMJ defects seen in smn mutants and blocking the intronic splice silencer site in exon 7 using antisense oligonucleotide therapy was able to increase human FL-SMN protein levels (Hao Le et al., 2011).
1.4.4 Mus musculus

The mouse *Smn* gene shares 82% coding sequence identity with human *SMN1* (Viollet et al., 1997). *Smn* was found to be vital during early embryonic development as *Smn*⁻⁻ embryos generated via exon 2 knockout led to cell death and failure in blastocyst transition (Schrank et al., 1997). Mice heterozygous for *Smn* null alleles, which have half normal levels of Smn protein, display a normal life span and are phenotypically normal until late in life when they develop mild motor neuron loss, axonal pathology and muscle weakness (Jablonka et al., 2000).

To understand the role of Smn in different tissues, various conditional knock-outs using the Cre-*loxP* system have been created. Neuronal restricted *Smn* exon 7 deletion lead to a SMA-like phenotype with progressive motor defects, signs of muscle denervation with neurofilament accumulation and lack of axonal sprouting at the NMJ despite no detectable motor neuron loss (Frugier et al., 2000, Cifuentes-Diaz et al., 2002). Skeletal muscle *Smn* exon 7 deletion in contrast results in severe muscular dystrophy which leads to motor impairment, paralysis and death after a median of 33 days (Cifuentes-Diaz et al., 2001). An adaptation of this model with restricted *Smn* depletion to differentiated myotubes (but not satellite cells) demonstrated improved motor performance and survival whereby non-mutated satellite cells were able to regenerate the muscle damage induced by *Smn* null mature fibres (Nicole et al., 2003). In the liver, homozygous deletion of *Smn* leads to late embryonic lethality due to a severe defect in liver development and iron overload (Vitte et al., 2004). These findings suggest a primary role of skeletal muscle and vital organs in SMA pathology alongside the denervation resulting from motor neuron involvement. However, as the presence of Smn is required for cellular viability (Schrank et al., 1997) it is perhaps not surprising that these conditional knock-out models display such severe phenotypes. Also as SMA is caused by low systemic levels of SMN and not complete absence of SMN in a specific tissue, these conditional knock-out mice do not accurately represent genetic models of SMA.

In order to more closely replicate the human condition, bacterial artificial chromosomes (BACs) containing the *SMN2* gene were added to mice with *Smn* null
alleles. In the ‘Taiwanese’ or ‘Hsieh-Li model, the addition of a SMN2 BAC transgene (denoted SMN2Hung, 115kb also containing SERF and part of NAIP) rescued the embryonic lethality of Smn−/− mice with hypoxanthine-guanine phosphoribosyltransferase (HPRT) knockout of Smn exon 7 (Hsieh-Li et al., 2000). As seen with human SMA patients, the resulting mice had a variable SMA-like phenotype depending on SMN2 copy number; Smn1hung−/−;SMN2Hungtg/0 mice with 2 copies of SMN2 had a severe phenotype with reduced body weight and surviving only 10 days where as Smn1hung−/−;SMN2Hungtg/tg mice with 4 copies of SMN2 had a mild phenotype of peripheral necrosis and normal life span (Hsieh-Li et al., 2000).

A second SMA mouse model, known as the ‘US model’, was published around the same time also showing rescue of embryonic lethality by addition of a 35.5 Kb SMN2 transgene to Smn1 mice previously generated by Schrank et al. (1997) (Monani et al., 2000b). As with the Taiwanese model, SMN2 acts as a modifier of disease severity in these mice (Monani et al., 2000b). Smn−/−; SMN2 mice with 2 copies of the transgene displayed a severe SMA-like phenotype with low body weight, lack of movement and motor neuron loss onset around postnatal day (P) 5 and death at P6 (Monani et al., 2000b). 8 copies of the transgene were sufficient to rescue this phenotype whilst mice with a single SMN2 copy are still born or die shortly after birth (Monani et al., 2000a, Monani et al., 2000b). The differences in survival times between the Taiwanese and US mice could possibly be due to strain differences, knockout construction (exon 7 vs exon 2) or size of the SMN2 transgene with SERF and NAIP potential modifiers of disease severity (Monani et al., 2000a).

A slightly slower progressing SMA model with mice carrying three SMN2 copies on a Smn null background has subsequently been created (Michaud et al., 2010). These mice have a median lifespan of 15 days with early NMJ defects in the diaphragm associated with reduced respiratory capacity (Michaud et al., 2010). Attempts to generate an intermediate mouse model of SMA have also been created whereby 0 to 8 copies of SMN2 were incorporated into the murine Smn1 locus by targeted mutagenesis (Osborne et al., 2012). Of the viable mutants, the adult Smn1C/C mouse with 2 copies of this human-mouse hybrid SMN2 allele and 2 copies of full length
human SMN2 displayed peripheral necrosis as well as a modest reduction in weight and motor performance (Osborne et al., 2012).

To further determine the role of SMNΔ7, mice carrying a SMNΔ7 transgene (SMN cDNA lacking exon 7) were crossed with the US SMA line to obtain a double transgenic Smn−/−;SMN2+/−;SMNΔ7+/− mouse model (Le et al., 2005). Addition of the SMNΔ7 transgene results in a milder phenotype and extended the life span of the US SMA mice from 5 to 13 days (Le et al., 2005). This result confirmed that SMNΔ7 is not a toxic product and that increasing SMNΔ7 is beneficial possibly due to the ability of FL-SMN and SMNΔ7 to associate, with oligomerisation stabilising SMNΔ7 turnover (Le et al., 2005). These mice demonstrate the classical neuromuscular symptoms of SMA including motor neuron loss and muscle atrophy over a more forgiving timeframe to study mechanisms of disease and evaluate therapeutic treatments (Le et al., 2005). Although not mimicking the human condition due to overexpression of the SMNΔ7 protein, the SMNΔ7 mouse is one of the most widely used models of SMA research. Adaptations have been made to the SMNΔ7 transgene to encode a more functional version of the protein to create a milder intermediate SMA model living 34 days on average (Cobb et al., 2013). Addition SMA models based on the severe US line have been created using the addition of transgenes containing human patient SMN1 point mutations to modulate phenotype severity (Monani et al., 2003, Workman et al., 2009). Both SMN1(A2G) and SMN1(A111G) are not sufficient to rescue Smn−/− mice but in the presence of SMN2, SMN1(A2G) and SMN1(A111G) mice display a mild phenotype and survive for around a year and are suggested to be used as model of Type III SMA (Monani et al., 2003, Workman et al., 2009). SMN1(A2G) mice exhibit mild motor neuron degeneration, muscle atrophy, axonal sprouting and abnormal electromyogram recordings (Monani et al., 2003). A similar neuromuscular phenotype was seen in the SMN1(A111G) mice with the phenotype severity correlated with snRNP assembly activity and snRNA levels (Workman et al., 2009).

A different approach to create a SMA mouse model has been to modify Smn into a humanised SMN2 like gene. An intermediate SMA model, termed Smn2B−/−, was
created by knock-in of the 2B allele which contains point mutations in the Smn exon splice enhancer site that binds the splicing factor Tra2-beta (2B) (Bowerman et al., 2009). The mutated 2B site disturbs Smn splicing resulting in production of an SMN2-like gene which produces Δ7Smn-like protein (Bowerman et al., 2009). The Smn<sup>2B/−</sup> mice have a median survival of around 30 days, however there is a broad variability of survival with around 20% of mice living over 300 days (Bowerman et al., 2012a). This variable phenotype may be due to their non-congenic mixed background as background strain is known to strongly influence the survival of SMA mice (Ackermann et al., 2013). Keeping Smn in its natural genomic context, a C-to-T mutation at the position corresponding to the nucleotide transition between SMN1 and SMN2 created a simpler genetic model of SMA (Gladman et al., 2010). These mice have a normal lifespan and exhibit a mild phenotype of muscle weakness, altered myofibre diameter and reduced locomotor activity making them a suitable model of Type IV SMA (Gladman et al., 2010).

Whilst mouse models have provided invaluable insight into the disease mechanisms underlying SMA they are not without limitations. The most widely used mouse models have very short lifespans which make it difficult to study underlying neurodegenerative mechanisms and also provide a very narrow therapeutic window for testing potential therapies. Unlike humans, the neonatal mouse blood-brain barrier (BBB) is relatively permeable indicating that therapies shown to directly target spinal motor neurons in mice may have limited translational applications. As reviewed in detail below, a large proportion of therapeutic strategies with beneficial outcomes in SMA mouse models have failed at clinical trials indicating that mice may have limited predictive validity for the human condition. In future, large animal models of SMA will be important in more closely recapitulating human disease progression and for the evaluation of biodistribution and efficacy of novel potential SMA therapeutics (Lorson et al., 2011).

1.4.5 Sus scrofa

Swine models are increasingly being used in translational research because of their similarity in anatomical and physiological characteristics to humans (Swindle et al.,
There is a 90% coding sequence identity between porcine and human SMN with pig Smn able to compensate for the loss of gem formation in human SMN1 deficient fibroblasts (Lorson et al., 2008). Knock-down of Smn in pigs using viral vector delivered RNA interference has enabled a swine model of SMA to be created (Duque et al., 2014). Intrathecal injection of virally delivered short hairpin RNA targeted against Smn in 5 day old piglets led to the development of SMA like symptoms, including a ~74% motor neuron loss and progressive hind limb muscle weakness, atrophy and fasciculations (Duque et al., 2014). However, in SMA reduced SMN levels is not just restricted to the CNS with non-motor neuron pathology playing an important contribution to SMA pathology (Hamilton and Gillingwater, 2013). Therefore, genetic disruption of Smn in pigs is also enabling the first large animal model of SMA based on the presence of human SMN2 to be created (Lorson et al., 2011). Smn gene targeting with single-stranded DNA and somatic cell nuclear transfer had enabled the first generation of SMN+/− piglets (Lorson et al., 2011). These Smn+/− piglets were healthy and phenotypically normal although no significant reduction in Smn mRNA was detected (Lorson et al., 2011) Importantly, splicing of SMN2 is conserved when introduced to pig cells, which following the creation of transgenic pigs carrying the human SMN2 transgene will enable the generation of a clinically relevant Smn−/−;SMN2 swine model of SMA (Lorson et al., 2011).

1.5 How low levels of SMN cause SMA

Although the genetic cause of SMA is well established it is not yet fully understood as why low levels of SMN protein lead to the distinctive SMA phenotype. As SMN is ubiquitously expressed, it also remains unclear why spinal motor neurons seem more susceptible to degeneration compared to other cell types. A number of different hypotheses have therefore been proposed to help explain the underlying SMA disease mechanisms (reviewed by (Burghes and Beattie, 2009)).

Due to SMN’s known role in mRNA splicing it has been proposed that reduced SMN levels affect snRNP assembly leading to an alteration in splicing of transcripts encoding proteins which are particularly important to motor neuron function.
(Burghes and Beattie, 2009). As discussed previously, studies from mouse modes show low levels of SMN reduce snRNP assembly activity, a defect that is correlated with SMA disease severity (Gabanna et al., 2007). snRNP assembly was found to be strongly decreased in both severe SMA and SMNΔ7 SMA compared to mild SMN1(A2G) SMA and control mice (Gabanna et al., 2007). Both neuronal and non-neuronal tissues were found to be affected although neuronal tissue was more affected with a 10-fold reduction in assembly activity in the brain and spinal cord compared to a 4-fold reduction in kidney tissues of severe SMA mice compared to controls (Gabanna et al., 2007). Interestingly, alterations in snRNPs in SMA mouse were found to be tissue specific with constituents of the minor spliceosome reduced in brain, spinal cord and heart whilst unchanged in muscle and kidney (Gabanna et al., 2007, Zhang et al., 2008). In addition, some components of the major spliceosome were increased in the heart, kidney and muscle (Zhang et al., 2008). These alterations in snRNP assembly as a driving cause of SMA have further been supported with evidence of numerous genes with altered splicing in SMA mouse models (Zhang et al., 2008). Exon array analysis revealed widespread tissue specific splicing of varied gene products (Zhang et al., 2008). Comparison of mRNA splicing of both severe and SMNΔ7 SMA mouse spinal cord tissue at different symptomatic time points revealed that the majority of splicing changes occur in at late symptomatic stages in pathways associated with neuronal development and cellular injury (Baumer et al., 2009). A small proportion of transcript changes were identified at a pre-symptomatic stage that may be important to eventual motor neuron survival (Baumer et al., 2009). However, it was unclear whether widespread splicing changes are responsible for SMA disease initiation or a consequence of disease progression (Baumer et al., 2009).

A more recent study using transcriptome profiling of laser captured motor neurons and adjacent white matter revealed selective and specific expression and splicing changes in motor neurons in pre-symptomatic severe SMA mice (Zhang et al., 2013). Around 3% of total genes analysed mRNA was affected, many of which are known to impair neurons, including Z exon skipping of agrin (an extracellular matrix protein critical for postsynaptic organisation of the NMJ) and alternative splicing of Gria2/4
mRNA which encodes AMPA glutamate receptor subunits important components of excitatory synapses (Zhang et al., 2013). Down-regulation of the neurotrophic factors Igf1/Igfr3 and the transcription factor Etv1/ER81 which plays an important role in sensory-motor neuron circuitry and up-regulation of C1q complement factor known to be involved in synapse pruning were also detected in SMA mouse motor neurons (Zhang et al., 2013). Such dysregulation of these specific motor neuron synaptogenesis genes preceding motor neuron loss could therefore provide a link between SMN deficiency and SMA’s signature pathology (Zhang et al., 2013). Together these studies show that low Smn levels in SMA mouse models leads to alterations in snRNP assembly and perturbations of pre-mRNA splicing causing profound changes in cellular RNA metabolism in a tissue-specific manner in SMA mouse models.

Another proposed hypothesis to explain the vulnerability of motor neurons in SMA lies in the mislocalisation of RNA to their distal axons (Rossoll et al., 2003). Motor neurons are unique in that their large metabolically active soma are linked to their muscular synaptic junctions by extremely long axonal processes with a robust neurofilamental cytoskeleton (Shaw and Eggett, 2000). The challenging transport of substances over such long axonal distances is thought be to one of the molecular mechanisms underlying the selective vulnerability of motor neurons in other neurodegenerative diseases such as ALS (Shaw and Eggett, 2000). The first evidence of SMN’s role in regulation of the cytoskeleton came when primary cultured motor neurons from severe SMA mice demonstrated a 30% reduction in axon growth compared to controls (Rossoll et al., 2003). The composition of the actin cytoskeleton in growth cones of SMA motor neurons is also dysregulated with an increase in the F-/G-actin ratio (Nolle et al., 2011). Reduced axon growth was correlated with β-actin mRNA and protein levels in distal axons and growth cones of cultured motor neurons with SMN or SMN’s RNA-binding protein binding partner hnRNP overexpression capable of promoting neurite growth in differentiating PC12 cells (Rossoll et al., 2003). However, in contrast to these in vitro studies and findings from lower animal models no defects in motor axon outgrowth have been found in mouse models of SMA in vivo (Mcgovern et al., 2008, Murray et al., 2010b).
Embryonic motor neuron outgrowth was shown to be normal in E10.5 severe US mice with motor axons developing and reaching the appropriate muscles at the same time as unaffected mice with denervation at later embryonic/early-postnatal stages (Mcgovern et al., 2008). The rate of motor axon growth during nerve regeneration following injury was unchanged in Smn<sup>2B−/−</sup> mice with injured nerves capable of re-innervating denervated endplates suggesting that there are no defects in motor axon pathfinding in SMA (Murray et al., 2013).

An alternative association between SMA and the cytoskeleton came when Smn was found to bind and co-localise the small actin-binding proteins profilin I and II in mouse motor neurons (Giesemann et al., 1999) and modulate actin polymerisation in vitro by reducing the inhibitory effect of profilin IIa (Sharma et al., 2005). Altered profilin II expression due to SMN depletion leads to up-regulation of the RhoA/ROCK pathway in differentiating PC12 cells, resulting in altered cytoskeletal integrity and a subsequent defect in neuritogenesis (Bowerman et al., 2007). SMN and profilin IIa-Smn binding is also severely impaired by the SMA patient S230L SMN2 missense mutation (Nolle et al., 2011). In SMA cell culture and tissue from the severe Taiwanese SMA mouse model there was differential phosphorylation of the ROCK-downstream targets cofilin, myosin-light chain phosphatase and profiling IIa (Nolle et al., 2011). Treating Smn<sup>2B−/−</sup> mice with a ROCK inhibitor was found to increase survival accompanied by an increase in muscle fibre size and maturation of NMJs (Bowerman et al., 2010). Furthermore, the actin bundling protein Plastin-3 is associated with SMN and can act as a protective modifier in some SMA patients (Stratigopoulos et al., 2010, Bernal et al., 2011, Oprea et al., 2008). Plastin-3 is involved in axogenesis by increasing the F-actin level with Plastin-3 overexpression rescuing axon length and outgrowth defects in zebrafish and in embryonic SMA mouse motor neuron cultures (Oprea et al., 2008). Knock-out of profilin II in Smn<sup>2B−/−</sup> mice is capable of upregulating Plastin-3 expression however this was not sufficient to rescue the SMA phenotype suggesting additional factors are involved in disease pathogenesis (Bowerman et al., 2009). Alongside the misregulation of actin dynamics, microtubule densities and β-III tubulin levels have also been shown to be reduced in distal axons of SMA mice with knock-down of stathamin, (a microtubule-
destabilising protein) promoting axon outgrowth and reducing mitochondrial transport defects in primary cultured motor neurons of SMA mice (Wen et al., 2010). Together these data suggest defects in axonogenesis are a major component of SMA and that increasing cytoskeleton stability may be therapeutically beneficial.

A defect in transport of RNA processing machinery or related mRNA transcripts along the axonal cytoskeleton due to low SMN levels, therefore, also could explain the cellular specificity of SMA (Rossoll et al., 2003). As discussed previously, SMN binds to many RNA-binding proteins, where it influences their bidirectional trafficking in axons and regulates the localisation and translation of mRNA (Akten et al., 2011, Sanchez et al., 2013, Rossoll et al., 2003). SMA associated SMN1 missense mutations can impair RNA-binding protein interaction and SMN deficiency also leads to reduction in RNA-binding protein levels and impaired mRNA axonal transport (Rossoll et al., 2003, Fallini et al., 2011). Impaired mRNA transport and local translation at growth cones and at the NMJ due to low SMN levels could therefore negatively affect motor neuron axon development, maintenance and regeneration (Fallini et al., 2012). Restoration of β-actin mRNA and protein in axons by increasing SMN protein levels in primary SMA mouse motor neuron cultures by blockade of cyclic AMP signalling rescued morphological and electrophysiological defects in growth cones (Jablonka et al., 2007). Restoration of β-actin levels in growth cones of SMN-deficient motor neurons can also be achieved by regulating protein synthesis by phosphatase and tensin homolog (PTEN) inhibition (Ning et al., 2010). PTEN is a tumour suppressor protein which negatively regulates the mammalian target of rapamycin (mTOR), an important promoter of protein synthesis and cell growth and has been shown to promote axon regeneration after injury (Park et al., 2010). Alongside the increased β-actin levels, in primary motor neuron cultures PTEN inhibition also causes changes in local protein synthesis and improved growth cone size, axonal elongation and increased cellular survival (Ning et al., 2010). PTEN gene silencing in SMNΔ7 SMA mice following hind limb muscle injection of adeno-associated viral (AAV) 6-siPTEN to achieve retrograde transduction of spinal motor neurons enhanced motor neuron survival and was able to ameliorate NMJ pathology (Ning et al., 2010, Little et al., 2015). Systemic
suppression of PTEN using self-complementary AAV (scAAV)9-siPTEN resulted in a 3-fold extension in lifespan of SMNΔ7 SMA mice indicating that PTEN can act an important SMA disease modifier (Little et al., 2015). Surprisingly, however, recent findings from a motor neuron specific β-actin knock-out mouse model showed that β-actin is not required for motor neuron viability or NMJ maintenance (Cheever et al., 2011). Aside from β-actin, other mRNAs have been shown to interact with SMN and RNA-binding proteins in complex and localise to axonal processes including candidate plasticity-related gene 15 (cpg15) (Akten et al., 2011). Cpg15, also known as neuritin, is known to promote motor neuron axon growth and neuromuscular synapase development (Javaherian and Cline, 2005). Cpg15 mRNA distribution and local axonal translation are perturbed in SMN-deficient neurons and cpg15 overexpression is capable of partially correcting the motor neuron axonal deficits observed in Smm-deficient zebrafish (Akten et al., 2011). The axonal and growth cone distribution of SMN and its RNA-binding protein interaction demonstrated in vitro using cell culture were recently confirmed in vivo in the axon terminals of embryonic and postnatal mouse motor neurons (Dombert et al., 2014). SMN and the RNA-binding protein hnRNP R were found to directly interact and co-localise in the embryonic mouse native spinal cord and NMJ at embryonic, postnatal and adult diaphragms suggesting SMN is important for recruitment and transport of RNA at all developmental stages (Dombert et al., 2014).

SMN may also modulate axonal local translation by its interaction with microRNAs (miRNAs); small non-coding RNAs which can act as regulators of post-transcriptional regulation of gene expression and induce RNA silencing (Kye et al., 2014). Reduction of Smn alters the level of miRNA expression and distribution in neurites of primary cultured neurons, in particular miR-183 whose increased levels were confirmed in the spinal cord of SMA mouse models (Kye et al., 2014). miR-183 regulated local axonal translation is reduced in Smn-deficient neurons and inhibition of miR-183 expression in Smm mutant mice improves survival and the motor phenotype (Kye et al., 2014). The RNA-binding proteins KSRP and FMRP, known to interact with SMN, have also been shown to play roles in miRNA function (Davis and Hata, 2009). Additionally, SMN can also associate with polyribosomes
and act as a translational regulator, a function that is abolished with disease associated SMN mutations (Sanchez et al., 2013). Combined, these studies provide a substantial body of evidence that SMN is involved in local mRNA regulation and whilst a lot more remains to be understood to fully appreciate the exact molecular mechanisms this body of work goes some way into insight to the basis of motor neuron death in SMA (Fallini et al., 2012).

1.5.1 How low SMN levels cause SMA: SMN and the ubiquitin proteasome system

As with many neurodegenerative disorders dysregulation of the ubiquitin proteasome system (UPS) has also been reported in SMA. The UPS regulates the specific degradation of proteins by the proteasome via a series of sequential enzymatic reactions (Figure 1-3). As reviewed in detail by Ciechanova and Brundin (2003), the first step involves ubiquitin being activated by an E1 activating enzyme resulting in a high energy thioester bond between the E1’s active site cysteine and the carboxyl group of ubiquitin in an ATP dependent manner. The activated ubiquitin is then transferred to an E2 conjugating enzyme by transthioesterification allowing the activated ubiquitin to be kept in a form that allows its transfer to a substrate protein (Ciechanover and Brundin, 2003). This final step is catalysed by E3 ubiquitin ligases that bind both the protein substrate and a specific E2 transferring ubiquitin to the target lysine residue resulting in the formation of an isopeptide bond (Ciechanover and Brundin, 2003). Polyubiquitin chains can build up on the target protein in this manner with some substrates requiring an additional E4 enzyme for elongation of ubiquitin chains (Ciechanover and Brundin, 2003). In humans 2 E1 enzymes (ubiquitin-like modifier activating enzyme 1 and 6 [UBA1 and UBA6]) are known, while there are 30-40 E2s and up to 1000 E3s which accounts for the exquisite substrate specificity of the UPS (Dennissen et al., 2012). Ubiquitination is also a reversible process with a large family of deubiquitinating enzymes (DUBs) regulating the removal of ubiquitin moieties from substrates and polyubiquitin chains (Ristic et al., 2014).
Abnormal granular ubiquitin deposits have been reported in ballooned anterior horn cells and higher brain structures of Werdnig-Hoffman disease patients (Matsumoto et al., 1993, Murayama et al., 1991, Lee et al., 1989, Kato and Hirano, 1990). These early neuropathology predate genetic testing, however a recent study of 5 Type I patients with confirmed SMN1 exon 7 deletion also showed similar deposits of ubiquitin in spinal cord neurons (Harding et al., 2015). Similar to ALS neuropathology, SMA motor neurons also stain highly for phosphorylated neurofilament but unlike ALS ubiquitin inclusions which are dense and well defined or filamentous ‘skein’ material (Leigh et al., 1991), SMA ubiquitin deposits are diffuse and granular like indicating that this pattern maybe unique to SMA. In 2008, the genetic cause of an X-linked form of SMA was identified as mutations in the UBA1 gene further implicating the UPS in infantile motor neuron diseases (Ramser et al., 2008).

Levels of SMN protein can be regulated by the UPS also making the UPS a potential therapeutic target for SMA (Chang et al., 2004). Inhibition of the proteasome (using MG132 or lactacystin) but not lysosomal, autophagy or calpain (a family of calcium dependent proteolytic enzymes) pathways lead to a dose-dependent increase in SMN levels and nuclear gems in cell lines and increased SMN levels in SMA patient-derived fibroblasts, indicating that the UPS is the main system that contributes to SMN degradation (Burnett et al., 2009). The highly specific FDA approved proteasome inhibitor bortezomib is also able to increase SMN levels in SMA patient fibroblasts (Kwon et al., 2011a). SMN is ubiquitinated prior to degradation (Chang et al., 2004) with ubiquitination of both FL-SMN and Δ7SMN markedly increasing rates of protein decay (Burnett et al., 2009). FL-SMN is predominantly conjugated to mono- and di-ubiquitin whereas SMNΔ7 is predominantly polyubiquitinated making it more susceptible to proteasome degradation (Han et al., 2012). SMN has been shown to interact with the E3 ligase mind bomb 1 (Mib1) with Mib1 overexpression in NSC34 cells increasing SMN ubiquitination and turnover (Kwon et al., 2013). Conversely, reducing Mib1 expression lead to increased SMN levels in cell culture with knock-down of the mib-1 orthologue in C.elegans ameliorating the pharyngeal...
Two DUBs which influence SMN levels have also been identified: ubiquitin specific peptidase 9, X-linked (Usp9x) (Han et al., 2012) and ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) (Hsu et al., 2010). Usp9x directly interacts with SMN with knock-down of Usp9x decreasing protein levels of SMN and the SMN complex (Han et al., 2012). Usp9x was found to deubiquitinate FL-SMN thereby protecting it from degradation, and ability which was abolished when the DUB activity of Usp9x was inhibited (Han et al., 2012). Interestingly, Usp9x did not regulate SMNΔ7 in a similar manner which may be due to the different cellular localizations of Usp9x (mainly cytoplasmic) and SMNΔ7 (predominantly nuclear) (Han et al., 2012). Usp9x also interacts with Mib1 (Choe et al., 2007) raising the possibility that they might work together to regulate SMN levels. The DUB UCHL1 also physically interacts with SMN is able to influence SMN expression (Hsu et al., 2010). In cell culture experiments, over-expression of UCHL1 reduced SMN levels and conversely pharmacological inhibition or UCHL1 specific knockdown significantly increased SMN expression (Hsu et al., 2010). Importantly, UCHL1 levels were found to be elevated in fibroblasts from SMA patients with levels correlated to disease severity with more UCHL1 protein in Type I patients than Type II and Type III (Hsu et al., 2010).

Recent breakthrough research has also identified an important role of SMN in the maintenance of ubiquitin homeostasis with alterations in the UPS a driving factor in SMA pathogenesis (Figure 1-3) (Wishart et al., 2014). Proteomic analysis of hippocampal synaptosomes of pre-symptomatic severe US SMA mice revealed widespread alterations in ubiquitin homeostasis in the neuronal cytoplasm including decreased levels of Uba1 and increased levels of Uchl1 (Figure 1-3) (Wishart et al., 2014). Significantly reduced levels of Uba1 were confirmed in the spinal cord and muscle of late-symptomatic US and Taiwanese mice illustrating that reduced Uba1 levels are an key molecular feature in the neuromuscular system in SMA mice (Wishart et al., 2014). Muscle Uba1 protein levels were also reduced at mid-
symptomatic time-point whereas, although spinal cord levels remained unchanged at this stage, the distribution of Uba1 in motor neurons was dramatically altered from a cytoplasmic to nuclear localisation (Wishart et al., 2014). Levels of monomeric and multimeric ubiquitin were also reduced in the spinal cords of US severe mice and were altered in their distribution in muscle of a Drosophila SMA model revealing that altered ubiquitination is an evolutionary conserved response to low SMN levels (Wishart et al., 2014).

SMN and UBA1 proteins were shown to physically interact and importantly, considering the role of SMN in mRNA splicing, the splicing of Uba1 mRNA was found to be altered in late-symptomatic Taiwanese SMA spinal cord (Wishart et al., 2014). Suppression of Uba1 in zebrafish using a morpholino or the pharmacological agent UBEI-41 led to SMA-like motor axon branching abnormalities with truncated or missing motor neurons present at high drug doses (Wishart et al., 2014). These zebrafish experiments illustrated that Uba1 suppression is sufficient to recapitulate a SMA motor-neuron phenotype in vivo indicating that reduced Uba1 activity directly contributes to SMA pathogenesis (Wishart et al., 2014). As a consequence of Uba1 reduction, levels of the downstream target of the UPS β-catenin, a dual function protein involved in cadherin cell adhesion and gene transcription by the Wingless/Wnt signalling pathway, were also found to be increased (Wishart et al., 2014). Under normal circumstances cellular β-catenin levels are tightly controlled by its ubiquitination and degradation by the proteasome, however the altered upstream UPS pathway in SMA means that β-catenin is no longer adequately degraded leading to its accumulation (Wishart et al., 2014). Suppression of Uba1 in cell-culture or zebrafish was found to be sufficient to greatly increase in β-catenin levels and signalling with the majority of proteins altered in SMA synaptosomes known to be β-catenin target genes (Wishart et al., 2014). β-catenin accumulation also found in the spinal cord of Taiwanese SMA mice and muscle biopsy samples from SMA patients revealing that altered β-catenin levels are a clinically relevant molecular perturbation in the SMA neuromuscular system (Wishart et al., 2014). Interestingly, β-catenin levels remained unchanged in the heart and liver of SMA mice revealing fundamental differences in the molecular mechanisms underlying organ and
neuromuscular pathology in SMA (Wishart et al., 2014). As discussed in detail later, β-catenin inhibition was capable of ameliorating the neuromuscular deficits in SMA mice (Wishart et al., 2014) proving that targeting this pathway is a promising therapeutic strategy for future studies.

**Figure 1-3. Schematic of the UPS.** Ubiquitin (Ub) is first activated by an E1 activating enzyme (e.g UBA1). The activated ubiquitin is transferred to an E2 conjugating enzyme, which binds an E3 ligase enzyme to transfer ubiquitin to a specific target substrate (eg. β-catenin). Once a polyubiquitin chain is built up on the target protein it is degraded into protein fragments by the 26S proteasome releasing free monomeric ubiquitin. Ubiquitin can also be removed from polyubiquitin chains by deubiquitinating enzymes (DUBs) (e.g. UCHL1). On the right, an overview of UPS alterations in SMA is outlined including a decrease in UBA1 levels and increase in β-catenin and UCHL1 levels. Image adapted from (Rahimi, 2012).
1.6 Therapeutic strategies for SMA treatment

1.6.1 SMN-dependent therapeutic strategies for SMA

Although there is no present cure for SMA, a number of promising advancements have been made in search of potential treatments. As low amounts of SMN cause SMA, the majority of therapeutic strategies under development are therefore aimed at trying to elevate SMN levels. As well as trying to restore functional SMN1 using gene therapy approaches, the presence of an intact SMN2 gene also makes an attractive target for SMA therapeutics (Lorson and Lorson, 2012). Although SMN2 ultimately only produces a small amount of the functional FL-SMN protein any intervention which would increase SMN2 efficacy, and therefore SMN protein production, would potentially ameliorate symptoms (Lorson and Lorson, 2012). Multiple possible intervention strategies have been investigated and aim to achieve this goal by increasing SMN2 transcription and translation, SMN2 transcript splicing correction, stabilisation of the SMN2 transcript or stabilisation of the SMN protein products (Lorson and Lorson, 2012).

1.6.1.1 Functional gene replacement: SMN gene therapy

Since SMA is monogenic and SMN cDNA is small, SMA is potentially well suited to viral replacement of SMN1 (Azzouz et al., 2004). The first study to show promising results of SMN gene therapy in SMA mice used a lentiviral vector to deliver SMN following multiple intramuscular injections (Azzouz et al., 2004). Injection of the lentivector equine infectious anaemia virus EIAV-SMN to hind-limb, respiratory and feeding muscles in P2 SMNΔ7 mice delayed weight loss onset by 2-3 days and increased mean survival by 5 days (Azzouz et al., 2004). Viral mediated gene expression in motor neurons at late-symptomatic stages indicated retrograde lentiviral SMN delivery with associated protection of loss of brainstem and spinal cord motor neurons (Azzouz et al., 2004). Although a minimal immune response was initiated in treated SMNΔ7 mice, serious safety concerns have been raised following trials of retrovirus gene therapy in humans (Yi et al., 2005). Although genome integration leads to strong expression it can also lead to insertional disruption of host
genes leading to oncogenesis with incidences of leukaemia reported in patients following retrovirus gene therapy treatment (Yi et al., 2005). Subsequent SMN gene therapy studies have therefore focused on using non-integrating adeno-associated virus (AAV) vector delivery.

Intravenous (IV) delivery of self-complementary AAV9 (scAAV9)-SMN at the day after birth in SMNΔ7 mice resulted in efficient transduction of the CNS including lumbar motor neurons (Valori et al., 2010). This was associated with improved phenotype including weight and motor performance comparable to control littermates up to P18 with a median survival of 69 days (Valori et al., 2010). After P18, rescued SMNΔ7 mice displayed a distinctive phenotype with shortened tails and necrotic ears similar to Type III Taiwanese mice associated with below normal SMN levels (Valori et al., 2010). In the same year, a similarly impressive rescue of the SMNΔ7 SMA mouse phenotype was reported by an independent group showing improvement of weight, survival and motor performance with and restored normal neuromuscular transmission with scAAV9-SMN treatment (Foust et al., 2010). Codon optimization of the human SMN1 sequence to enhance expression in mouse tissues coupled with a strong promoter further substantially increased mean life expectancy to 160 days following a single IV delivery of scAAV9-SMN (Dominguez et al., 2011). Importantly it was found that whilst scAAV9-SMN delivery at P2 robustly corrected the SMA phenotype, delivery at P5 resulted in partial correction whilst delivery at P10 had little effect illustrating a therapeutic window at earlier developmental stages (Foust et al., 2010).

As well as IV scAAV-SMN delivery, CNS targeted delivery has also shown improvements in SMA mouse models. AAV8-SMN delivery in to both cerebral lateral ventricles and the upper lumbar spinal cord in newborn SMNΔ7 mice results in widespread increase in SMN levels throughout the spinal cord and improved skeletal muscle pathology (Passini et al., 2010). scAAV8-SMN lead to an improvement of gene therapy efficacy, increasing survival to a median of 157 days compared to 50 days with single-stranded AAV8 (Passini et al., 2010). In a comparison of IV and intracerebroventricular (ICV) scAAV9 delivery, both injection
routes lead to phenotypic improvements in SMNΔ7 mice with the ICV scAAV9 treated mice gaining weight more efficiently and displaying fewer early deaths than the IV treated group (Glascock et al., 2012b). These findings were replicated in the more severe US model demonstrating that route of delivery is an important consideration with SMN gene therapy (Glascock et al., 2012a). In pigs, intrathecal injection of scAAV9-shRNA resulted in a 75-80% knockdown of Smn mRNA in the spinal cord causing ~80% motor neuron loss and hind limb muscle wasting (Duque et al., 2014). In this model scAAV9-SMN intrathecal gene therapy delivered pre-symptomatically prevented the development of the SMA phenotype while delivery at the onset of symptoms resulted in a marked phenotypic improvement including prevention of motor neuron loss and electrophysiological neuromuscular defects (Duque et al., 2014). This first illustration of the therapeutic potential of scAAV9-SMN gene therapy in a large animal model of SMA is encouraging for clinical translation (Duque et al., 2014).

The clinical potential of scAAV9 gene therapy for SMA was further emphasized when IV injection of scAAV9-GFP into a newly born macaque showed that scAAV9 could cross the BBB and transfet motor neurons in non-human primates (Foust et al., 2010). However, the presence of high levels of AAV neutralising antibodies that can significantly lower gene transfer in humans compared to other species is a significant obstacle of AAV gene therapy development (Rapti et al., 2012). Technical limitations also come into play with current vector production technologies unable to manufacture the volumes of virus to accommodate large scale clinical trials for systemic delivery in SMA patients although trials requiring smaller viral volumes may be feasible (Lorson and Lorson, 2012). Indeed a clinical trial to evaluate safety and efficacy of intravenous scAAV9-SMN gene transfer is currently recruiting SMA Type I patients with an estimated study completion date of June 2017 (Clinicaltrials.Gov, 2014).
1.6.1.2 Targeting SMN2 promoter activation: histone deacetylase inhibitors

One of the most studied pharmacological treatment options for SMA is the use of histone deacetylase inhibitors (HDACis) to increase SMN protein levels via SMN2 promoter activation (Lunke and El-Osta, 2013, Mohseni et al., 2013). HDACis act to modulate chromatin structure by inhibiting HDAC activity; thereby unwinding DNA from histones making it more accessible to transcriptional machinery. HDACs also have non-histone targets which include transcription factors and cell signalling pathways, meaning that HDACis can also influence gene expression via non-canonical chromatin modification roles, and can also influence mRNA and protein stability (Singh et al., 2010). In relation to SMA therapy, HDACis are effective in increasing FL-SMN levels through a mechanism most likely linked to chromatin remodelling of the SMN2 promoter region (Kernochan et al., 2005) but also by influencing non-histone HDAC activity (Hauke et al., 2009) and SMN2 exon 7 alternative splicing correction (Chang et al., 2001, Sumner et al., 2003, Brichta et al., 2003, Harahap et al., 2012).

In 2001, sodium butyrate was the first HDACi to show therapeutic potential for SMA both in vitro and in vivo (Chang et al., 2001). In SMA patient cell lines, sodium butyrate successfully altered the splicing processing of SMN2 transcripts and was able to effectively increase FL-SMN protein levels (Chang et al., 2001). In Type II and III SMA mice, sodium butyrate treatment increased SMN levels in lower motor neurons and improved the disease phenotype whilst oral treatment of pregnant dams reduced the severity of the SMA offspring (Chang et al., 2001). The pharmacokinetics of sodium butyrate are well understood and toxicity is low in humans and animal models but a short serum half-life leads to a lack of clinical efficacy in patients (Miller et al., 1987).

Further investigation of related compounds was shortly conducted. In 2003, valproic acid (VPA) was shown to increase SMN2 promoter activation, restore splicing patterns, increase SMN2 mRNA/FL-SMN protein and gem number in vitro (Sumner et al., 2003, Brichta et al., 2003, Piepers et al., 2011, Harahap et al., 2012). In vivo,
VPA increased FL-SMN protein levels in the spinal cord of Type III-like SMA treated mice and was associated with multiple beneficial effects, including improved motor function, decreased motor neuron degeneration, reduced muscle atrophy and NMJ denervation (Tsai et al., 2008a). VPA treated SMA mice also showed increased levels of levels of anti-apoptotic spinal cord proteins and signs of induced neurogenesis indicating a neuroprotective effect (Tsai et al., 2008a). Other studies, however, have found however that VPA can block excitability in SMA mouse motor neuron culture by reducing growth cone size and inhibiting voltage-gated calcium channels, potentially aggravating SMA disease specific symptoms (Rak et al., 2009).

Of 20 SMA patients treated with VPA in a pilot study, roughly half the volunteers demonstrated increased SMN2 mRNA and SMN protein blood levels (Brichta et al., 2006). In a longer 8-month, open-label study of 7 Type III/IV SMA volunteers, VPA treated patients had demonstrated increased quantitative muscle strength and subjective function (Weihl et al., 2006). In a larger open-label pilot study of 42 SMA type I-Ill patients VPA treatment was shown to improve gross motor function of younger Type II non-ambulatory children (Swoboda et al., 2009). These promising preliminary reports, led the way for larger controlled trials to be conducted. In 2010, a randomized, double blind, phase II study was conducted in both in 61 Type I non-ambulatory children and 33 Type II ambulatory patients treated with VPA and L-carnitine for 6 months, however no significant improvements in strength and function were found in either cohort (Swoboda et al., 2010, Kissel et al., 2011). A later 12-month, double-blind, cross-over study of VPA in ambulatory 33 SMA adults also showed no beneficiary impact (Kissel et al., 2013). These disappointing results were further confounded by the adverse effects of VPA, including severe carnitine depletion and debilitating weight gain with the associated lean mass loss reducing gross motor performance in some patients (Tsai et al., 2007, Swoboda et al., 2009, Swoboda et al., 2010, Kissel et al., 2011).

Similar in vitro and in vivo results to VPA have been observed with sodium phenylbutyrate (PB) (Lunke and El-Osta, 2013). PB was found to increase FL-SMN levels and gem numbers in SMA patient fibroblasts with Type II and III derived cells.
responding more to disease treatment than Type I cells (Andreassi et al., 2004). A 9-week pilot trial of PB in 10 Type II patients illustrated a preliminary improvement in motor performance (Brahe et al., 2005). However, in a large, randomized, double blind, placebo-controlled trial of 107 SMA patients, PB was not effective at improving motor symptoms (Mercuri et al., 2007). Criticisms of this study’s intermittent dosing regimen may have negated any beneficial impact of the drug with a trial of continuous PB treatment currently ongoing (Lunke and El-Osta, 2013). PB is FDA approved to treat urea cycle disorders, however as with VPA, high doses are required with poor compliance due to numerous adverse effects (Leonard and Morris, 2002).

Due to the issues outlined above the search for more potent, second-generation HDACi ensued. The FDA approved suberoylanilide hydroxamic acid (SAHA) is able to activate the SMN2 gene and increase FL-SMN protein levels in SMA patient fibroblasts, motor neuron cell cultures and human brain tissue at lower concentrations than VPA and PB (Hahnen et al., 2006). In vivo data has demonstrated that SAHA treatment of pregnant dams increases viability of severe US-SMA mouse offspring (Riessland et al., 2010) and can increase life expectancy by ~30%, improve motor performance, counteract motor neuron loss, increase NMJ size, increase muscle size and can increase neuromuscular SMN levels in the Type III Taiwanese SMA model (Riessland et al., 2010). Vascular defects in SMA mice can also be ameliorated with SAHA (Somers et al., 2013). Similarly, trichostatin A (TSA) has also been shown to increase SMN protein and SMN2 transcript levels in neural and muscle tissues with an improvement of snRNP assembly in SMA mice (Avila et al., 2007). TSA can improve survival, attenuate weight loss, improve motor performance and increase myofibre and motor neuron size even when administered after disease onset (Avila et al., 2007). TSA administered mice also show increased muscle innervation (Ling et al., 2012), partially restored impaired spinal reflexes (Mentis et al., 2011), and sustained improvement when coupled with nutritional support (Narver et al., 2008).
1.6.1.3 Targeting SMN2 promoter activation: other small molecule therapeutics

A high-throughput bioassay identified quinazoline-based compounds as the most potent SMN2 promoter inducers, being able to robustly increase SMN levels and gem numbers in SMA patient fibroblasts (Jarecki et al., 2005, Thurmond et al., 2008). The lead compound 2,4-diaminoquinazoline was found to function by inhibiting the scavenger mRNA decapping enzyme DcpS which is a known modulator of RNA metabolism (Singh et al., 2008). Oral administration of the 2,4-diaminoquinazoline derivative D156844 increased SMN2 promoter activity and SMN levels in the mouse CNS improving the motor performance and survival of SMNΔ7 mice (Butchbach et al., 2010). Another quinazoline compound RG3039 was found to be well tolerated at doses that robustly inhibit DcpS in the CNS in SMNΔ7 mice although SMN expression and snRNP assembly were only minimally enhanced (Van Meerbeke et al., 2013). Despite this, RG3039 increased survival and motor performance with improved motor neuron synaptic input and prevention of NMJ denervation (Van Meerbeke et al., 2013). Improved survival, motor performance and motor unit pathology was also seen in RG3039 treated Taiwanese and Smn2B/- SMA mice (Gogliotti et al., 2013). RG3039 has progressed to Phase I clinical trials where it was found to be safe, well-tolerated and effective at inhibiting DcpS in healthy volunteers (Repligen, 2010).

SMN2 activation can also be effectively enhanced by inducing STAT5 (signal transducers and activators of transcription 5) activation (Ting et al., 2007). Transcriptional regulation following STAT5 activation enhances SMN2 promoter activity resulting in an increase in FL-SMN and SMNΔ7 transcripts in motor neuron-like NSC34 cells (Ting et al., 2007). Constitutive activation of STAT5 increases nuclear gem number in SMA patient cells and diminishes axon growth defects in SMA-like motor neurons (Ting et al., 2007). Inducing STAT5 activation in SMA mouse models has been shown to ameliorate the disease phenotype (Liu et al., 2013, Farooq et al., 2011). Sodium vanadate improved the motor performance and delayed disease progression of muscle atrophy and peripheral necrosis in the mild Type III Taiwanese mouse model (Liu et al., 2013). Prolactin, another STAT5 inducer,
strongly upregulates SMN mRNA and protein levels in the brain and spinal cord of SMAΔ7 mice which correlated with improved weight, motor performance and an increased mean survival from 13 to 21 days (Farooq et al., 2011). The lack of prolactin receptor expression outside of the CNS meant that SMN levels were not increased in peripheral organs which could help to explain the still early mortality despite strong CNS expression of SMN (Farooq et al., 2011). Both sodium vanadate and prolactin, however, are associated with toxicity which may prevent these compounds moving forward to clinical trials in SMA patients (Liu et al., 2013, Farooq et al., 2011).

The anti-cancer ribonucleotide reductase inhibitor hydroxyurea has been shown to increase SMN2 gene expression and nuclear gem numbers in cells from Type I-III SMA patients (Grzeschik et al., 2005). This effect was later found to be mediated by the hydroxyurea metabolite nitric oxide with other nitric oxide donors also producing similar effects on SMN2 expression (Xu et al., 2011). Spinal cord SMN protein levels were also increased in heterozygous US SMA mice following hydroxyurea treatment (Mattis et al., 2008b). A pilot study of hydroxyurea therapy in 33 Type II- III SMA patients revealed no significant improvement in functional motor assessment although a slight increase in SMN mRNA levels and manual muscle test scores were reported (Liang et al., 2008). A larger randomized, double-blind, placebo-controlled trial of 55 Type II- III patients, however, found no improvement in strength and motor function with a significant number of patients also developing neutropenia due to hydroxyurea’s effect on bone marrow suppression (Chen et al., 2010b).

1.6.1.4 Targeting SMN2 splicing: antisense oligonucleotides

Antisense oligonucleotide (ASO) therapy for SMA is designed to increase exon 7 inclusion of SMN2 thereby increasing the amount of translated FL-SMN protein. ASOs are short stretches of nucleic acid that hybridize to mRNA target sequences modulating gene expression (Dias and Stein, 2002). ASOs can be divided into two main categories by their mechanisms of action: 1) RNAase H dependent ASOs which degrade mRNA by inducing cleavage by ribonuclease H or 2) steric blocker ASOs which physically prevent the progression of splicing or translational
machinery (Dias and Stein, 2002). It is the latter steric blocker ASOs which have been utilized with great effect to alter SMN2 pre-mRNA splicing (Porensky and Burghes, 2013). SMN2 mRNA has a number of local and regional splice silencers and enhancers which can be targeted to increase exon 7 retention (Porensky and Burghes, 2013). Conversely, ASOs that prevent SMN2 exon7 inclusion can also be used to generate mouse models of SMA (Sahashi et al., 2012, Sahashi et al., 2013).

One of the simpler strategies is to use ASOs to target intronic splice silencer (ISS) elements surrounding exon 7 which inhibit SMN2 exon7 inclusion (Lorson and Lorson, 2012). ISS-N1 is located immediately downstream of the 5’ splice site in intron 7 with ISS-N1 deletion or targeting by ASO promoting exon 7 inclusion and increasing SMN levels in SMA patient cells (Singh et al., 2006). The same ASO is also capable of modifying SMN2 splicing and increasing FL-SMN protein levels in the spinal cord of SMNΔ7 mice following ICV delivery with associated improvements in weight and motor function (Williams et al., 2009). Splicing repression at the ISS-N1 site was found to be mediated by the RNA-binding proteins hnRNP A1 and A2 with mutations or blocking ASOs against hnRNP A1/A2 also promoting efficient exon 7 inclusion (Hua et al., 2008). Examination of the two lead ASOs: 10–27 and 09–23 in SMN2 transgenic mice revealed that IV delivery also corrected SMN2 splicing in vivo with increased exon 7 inclusion detected in the liver, kidneys and muscle but not spinal cord as ASOs were incapable of crossing the BBB (Hua et al., 2008). ICV infusion of ASO-10-27 in adult Type III Taiwanese mice increased spinal cord SMN2 exon 7 inclusion and SMN protein levels in a dose dependent manner (Hua et al., 2010). SMN levels remained increased for up to 2 months following a single ASO-10-27 ICV injection and was able to reduce their necrotic tail and ear phenotype (Hua et al., 2010). ICV injection of ASO-10-27 in SMAΔ7 mice at the day of birth was able to increase exon 7 inclusion and SMN levels in the spinal cord with improvements in neuromuscular pathology and a mean extension in survival from 14 to 26 days (Passini et al., 2011). Systemic delivery of ASO-10-27 following subcutaneous injection was much more effective at improving the SMAΔ7 phenotype with an impressive mean 25-fold extension in lifespan indicating that widespread targeting of organs by ASO therapy would be necessary.
for long-term rescue of SMA pathology (Hua et al., 2011). Indeed, ICV injection of a
decoy oligonucleotide to neutralise the CNS effects of ASO-10-27 following
subcutaneous delivery did not negatively impact on the rescue of tail necrosis in the
mild Type III Taiwanese model (Hua et al., 2015). Strikingly, CNS neutralization by
the decoy oligonucleotide also had no negative impact on the improvement in
survival of severe Type I Taiwanese mice (Hua et al., 2015). Decoy treatment also
had no impact on weight, motor performance, NMJ integrity or motor neuron counts
even though motor neuron SMN levels and gem counts were reduced (Hua et al.,
2015). This study importantly shows that SMA is not a cell-autonomous defects of
motor neurons and that peripheral SMN restoration of SMN levels is sufficient for
full phenotypic rescue of SMA mice (Hua et al., 2015).

ISIS Pharmaceuticals have developed the back bone of ASO-10-27 to create an ASO
SMA therapy called ISIS-SMNRx which has progressed into clinical trials. An
ongoing open-label Phase II clinical studies of ISIS-SMNRx in Type I infants has
shown that a single or repeated intrathecal doses is well tolerated and was able to
increase muscle function scores (Thesmatrust, 2014). Analysis of postmortem patient
tissue showed that ISIS-SMNRx following lumbar puncture delivery was distributed
throughout the spinal cord including in motor neurons where SMN2 exon 7 inclusion
was increased as were SMN protein levels (Thesmatrust, 2014). Following these
promising results, ISIS Pharmaceuticals are now recruiting for two Phase III
randomized, double-blind, sham-procedure controlled studies in approximately 100
infants diagnosed with either infant- or later-onset SMA (Thesmatrust, 2014). It
remains to be seen if ISIS-SMNRx therapy will be beneficial in late-onset SMA
considering the established temporal requirement of restoring SMN levels in SMA
mouse models. It also remains to be seen if intrathecal delivery with the aim of
increasing motor neuron SMN levels is the best delivery approach considering the
peripheral organ defects also reported in SMA patients. It may be that peripheral
ASO injections or a combinatory peripheral and CNS delivery approach may yield
better therapeutic outcomes.
1.6.1.5 Targeting *SMN2* splicing: small molecule therapeutics

As well as ASOs, other small molecule therapeutics can also correct *SMN2* splicing. An early study which screened for compounds that increased cellular levels of FL-SMN from the *SMN2* gene identified acrurubicin as a lead hit (Andreassi et al., 2001). Acrurubicin treatment resulted in a 4-fold increase in the ratio of FL- to Δ7-SMN in Type I SMA patient fibroblasts and increased nuclear gem numbers (Andreassi et al., 2001). A screen using a cell-free splicing system to focus on compounds that modulate splicing in the absence of other potential cellular modifiers of gene expression identified the antibiotic tetracycline derivative PTK-SMA1 as a unique therapeutic candidate (Hastings et al., 2009). PTK-SMA1 was found to stimulate splicing from *SMN2* exon 6 to exon 7 and block *SMN2* exon 7 skipping, increasing FL-SMN protein levels in SMA patient fibroblasts and both mild Taiwanese and severe US SMA mouse models (Hastings et al., 2009).

Salbutamol, the β2-adrenergic receptor agonist used in asthma treatment, was found to rapidly and efficiently increase FL-*SMN2* transcripts in a SMA fibroblast cell line (Angelozzi et al., 2008). Salbutamol also increased SMN protein levels and nuclear gem numbers *in vitro* (Angelozzi et al., 2008). 12 Type II-III patients who took salbutamol for a year had significantly elevated *SMN2* full length transcript levels in blood leukocytes which was correlated to *SMN2* copy number, demonstrating clinical molecular efficacy (Tiziano et al., 2010). In a pilot study, 6 month salbutamol treatment significantly improved maximal voluntary isometric muscle contraction, lung function and lean body mass in 13 Type II-III SMA patients although overall muscle strength remained unchanged (Kinali et al., 2002). Salbutamol was well tolerated although hand tremors were noted in a significant proportion of patients (Kinali et al., 2002). In a larger cohort of 23 Type II SMA patients, daily salbutamol over a 1 year period significantly improved motor functional scores compared to base line readings indicating that larger randomized, double-blind, placebo controlled trials may be warranted (Pane et al., 2008).

A high-throughput screen of a small molecule library identified several classes of compounds that could promote *SMN2* exon 7 inclusion (Naryshkin et al., 2014).
After optimisation, 3 orally available compounds were identified (SMN-C1, SMN-C2, and SMN-C3) which could increase SMN2 exon 7 inclusion and increase FL-SMN levels in Type I SMA patient fibroblasts and motor-neuron patient-derived induced pluripotent stem cells (iPSCs) (Naryshkin et al., 2014). RNA sequencing revealed that cells treated with SMN-C3 did not induce widespread changes in gene expression indicating that these novel SMN splicing modifiers are highly specific (Naryshkin et al., 2014). In the mild adult Smn1/C-C SMA mouse model a single oral dose of SMN-C3 SMN2 full length mRNA transcripts increased from 40% to 90% of total SMN transcripts with SMN protein levels increasing by up to 70% in the brain and quadriceps muscle (Naryshkin et al., 2014). Oral dosing over 10 consecutive days resulted in a 200% increase in SMN levels with FL- and Δ7-SMN mRNA returning to baseline within 32 hours of stopping treatment (Naryshkin et al., 2014). In SMNΔ7 mice daily SMN-C3 delivery resulted in improved weight, motor performance, neuromuscular pathology and an increased median survival from 18 to over 65 days (Naryshkin et al., 2014). This identification or orally bioavailable SMN2 splicing modifiers that target both the CNS and peripheral tissue is encouraging for further investigation as a promising SMA therapeutic (Naryshkin et al., 2014). However, in 2015 clinical trials in SMA patients are currently on hold due to unexpected findings in long-term primate studies (Cure-Sma, 2015).

1.6.1.6 Promoting SMN stabilisation

A screen of compounds capable of increasing cellular SMN levels found that inhibition of glycogen synthase kinase 3 (GSK-3) caused increased levels of SMN by protein stabilisation (Makhortova et al., 2011). GSK-3 was found to phosphorylate SMN and decrease its levels by increasing its turnover rate (Makhortova et al., 2011). GSK-3 inhibitors therefore reduce the rate of SMN degradation and are able to prolong survival of embryonic stem cell derived motor neurons with low levels of SMN (Makhortova et al., 2011). In SMNΔ7 mice the GSK-3 inhibitor BIP-135 was able to modestly increase survival by 2 days (Chen et al., 2012a).

Aminoglycosides have been shown to increase SMN levels in SMA patient fibroblasts by promoting SMN stability by promoting read-through of the SMNΔ7
protein (Mattis et al., 2008a). Suppressing recognition of stop codons by aminoglycosides can induce a longer SMNΔ7 protein C-terminus (Wolstencroft et al., 2005). Extension of the C-terminus of the SMNΔ7 protein by translational read-through can confer functionality by increasing snRNP assembly and promoting neurite outgrowth in SMN deficient cells (Mattis et al., 2008a). Treatment of SMNΔ7 mice with the FDA approved aminoglycoside G418 increased SMN levels and increases motor function although chronic administration was associated with toxicity (Heier and Didonato, 2009). ICV injection of the aminoglycoside TC007 increased brain SMN protein levels, improved motor unit pathology and function with an increased lifespan from 13 to 16 days (Mattis et al., 2009a). Sub-cutaneous TC007 delivery was also associated with beneficial effects on muscle fibre size and gross motor function (Mattis et al., 2009b).

SMN mRNA stability has also shown to be increased by the antibiotic anisomycin (Farooq et al., 2009). Ansisomycin causes activation of the p38 mitogen-activated protein kinase (MAPK) pathway inducing cytoplasmic accumulation of the RNA-binding protein HuR which in turn binds to the 3’-untranslated region of SMN causing its stabilization (Farooq et al., 2009). An upstream activator of p38 MAPK, apoptosis signal-regulating kinase 1 (ASK1) also stabilizes SMN with depletion of ASK1 mimicking the neurite outgrowth defects seen in SMN-deficient cells (Kwon et al., 2011b).

1.6.2 SMN-independent therapeutic strategies for SMA treatment

Whilst many of the SMN-dependent therapies show exciting promise, as outlined above a number of limitations with current approaches exist. As discussed previously, there is also a well-defined therapeutic window in early development where SMN restoration is required and after which no beneficial impact is seen (Le et al., 2011, Foust et al., 2010). Considering this temporal requirement of restoring SMN levels in SMA animal models it remains to be seen if SMN therapy is capable of success in SMA patients which are not diagnosed immediately after birth. All SMN-dependent therapies in clinical trials have shown no or limited effect,
particularly for adolescent Type III patients. It may be that foetal SMN replacement may be required in utero to achieve maximum therapeutic potential of these approaches although universal prenatal screening for SMA is not currently carried out as it is not deemed cost-effective (Little et al., 2010). A number of SMN independent therapeutic approaches exist which have been shown to reduce motor neuron degeneration or increase muscle strength in SMA models (reviewed by (Tsai, 2012)). These strategies aim to find ways to prevent SMN deficient motor neurons from degenerating independent of trying to increase SMN protein. Providing SMN-independent therapeutics could help to improve motor and peripheral organ function in SMA patients even if SMN itself isn’t restored.

In addition to previously discussed SMN independent strategies targeting axonal dynamics (profilin, plastin-3, RhoA/ROCK), or protein synthesis (PTEN), the following non-classical targets may also provide alternative or adjunctive strategies for SMA therapy.

**1.6.2.1 Stem cells: motor neuron replacement therapy**

As spinal motor neuron loss will have already occurred by the time a patient is diagnosed with SMA, stem cell therapy may provide a possibility of motor neuron cell replacement (Tsai, 2012). Foetally derived neural stem cells (NSCs) are self-renewing and multipotent and are capable of differentiating into motor neurons in vitro when grown in the presence of retinoic acid and sonic hedgehog after priming with specific growth factors (Corti et al., 2008). To test the therapeutic potential in SMA a subset of NSCs were intrathecally implanted into P1 SMNΔ7 mice (Corti et al., 2008). Using Hb9-GFP mice as donor NSC cells allowed visual confirmation that donor cells were able to migrate throughout the spinal cord and engraft into the ventral horns of SMA mice, although they only represented 3.5% of total motor neuron numbers (Corti et al., 2008). NSC transplantation therapy modestly improved weight and motor performance of SMNΔ7 mice and increased mean survival time from 13 to 18 days (Corti et al., 2008). Improvements in motor neuron loss and muscle fibre diameters were also seen (Corti et al., 2008). Considering the low number of donor motor neurons and their limited 2-3mm axonal outgrowth length
the authors suggested it was unlikely that this amelioration of disease phenotype was due to restoration of functional motor units (Corti et al., 2008). Microarray analysis of isolated motor neurons revealed that neuroprotection in NSC treated SMNΔ7 mice was likely due to modulation of the gene expression profile to a more wild-type like state (Corti et al., 2008). In vitro analysis showed that NSC co-culture can upregulate the expression of neurotrophins and growth factors which increase SMA motor neuron axon length indicative of NSCs supplying trophic support to surrounding cells (Corti et al., 2008).

Although this study showed promise for NSC therapy in a SMA mouse model any translation to patients is unlikely due to the limited availability of adult CNS tissue to create human NSCs alongside ethical and safety issues (Corti et al., 2010). Embryonic stem cells (ESCs) derived from the inner cell mass of a blastocyst, may provide a more readily available source of pluripotent stem cells that are also able to differentiate in to motor neuron like cells in vitro (Corti et al., 2010). Transplantation of NSCs derived from an ESC cell line into the cerebrospinal fluid of SMNΔ7 mice show extensive migration in the spinal cord where they differentiate in to motor neurons and other neuronal types (Corti et al., 2010). Improvements in weight and functional motor recovery were seen following transplantation of ESC-derived NSCs with an increased mean survival from ~12 to 20 days (Corti et al., 2010). As seen with primary NSC implantation, ESC-derived NSCs promoted neuroprotection in vivo although there was no evidence of newly generated motor neuron outgrowth to muscles (Corti et al., 2010).

Induced pluripotent stem cells (iPSCs) were first generated by reprogramming of either embryonic or adult somatic cells into a pluripotent state by expressing four specific transcription factors under embryonic cell culture conditions (Takahashi and Yamanaka, 2006, Yu et al., 2007). Human iPSCs (hiPSCs) have since been generated from skin fibroblast samples taken from Type I SMA patients (Ebert et al., 2009, Chang et al., 2011). These SMA hiPSCs have been differentiated into motor neuron-like cells that express markers for mature motor neurons (Ebert et al., 2009). At 8 weeks post-differentiation equal numbers of hiPSC SMA and wild-type motor
neurons were seen, however, after two weeks in culture hiPSC motor neurons were fewer and smaller than wildtype (Ebert et al., 2009). Reduced motor neuron production has been reported in hiPSC lines from an independent Type I patient which also displayed slower neurite development (Chang et al., 2011). hiPSC SMA motor neurons show increased activation of cell death pathways markers compared to control with inhibition of apoptosis preventing motor neuron death seen in SMA hiPSC cultures (Sareen et al., 2012). A down-regulation and differential splicing of genes encoding protein involved in neuronal and synaptic development have also been detected in SMA-hiPSC motor neurons (Corti et al., 2012). Drugs previously reported to increase SMN levels in primary fibroblasts and SMA mouse models also increased SMN protein levels and gem numbers in hiPSC SMA motor neurons indicating that these cells could be used to screen drugs specifically on human motor neuron like cells (Ebert et al., 2009). Genome editing of SMA hiPSCs by oligonucleotides to convert SMN2 to a SMN1 like gene by exon 7 incorporation enabled genetically corrected motor neurons to be generated (Corti et al., 2012). Engraftment of these corrected SMA hiPSC motor neurons into SMNΔ7 mice spinal cords ameliorated their phenotype with improved motor performance and an increased mean life expectancy from 14 to 21 days (Corti et al., 2012). Donor motor neurons represented 27.5% of the total motor neuron population and endogenous motor neurons were also increased by 6.8% in the hiPSC treated group (Corti et al., 2012). Muscle area was also increased and unlike NSC or ESC therapy a few donor motor neurons were able to extend to the periphery and form new NMJs with skeletal muscle (Corti et al., 2012). The use of corrected SMA-hiPSC–derived motor neurons also offers an ethical advantage over NSCs and ESCs as well as being able to use autologous cells from patients reducing the possibility of immune rejection (Corti et al., 2012).

Although the translation potential of stem cell replacement therapy for SMA is currently low, future work to enhance motor function following stem cell transplant in SMA mice could make use of compounds to overcome myelin mediated repulsion and addition of neurotrophic factors in distal nerves attract stem-cell derived axons toward skeletal muscle targets (Deshpande et al., 2006). Nevertheless hiPSCS also
provide useful models to understand the molecular pathogenesis underlying motor neurons derived from patient cells and also as drug screening platforms.

1.6.2.2 Trophic factors

Congenital heart abnormalities are a feature of severe SMA with up to 75% of patients with a single SMN2 copy presenting with major cardiac septal defects (Rudnik-Schoneborn et al., 2008, Moller et al., 1990, Distefano et al., 1994). Functional heart defects are also present in SMA mice (Heier et al., 2010, Shababi et al., 2010). Systemic delivery of cardiotrophin-1, a cardiac hypertrophic cytokine, in a SMA mouse model of SMA with a neuronal specific Smn exon 7 exclusion had neurotrophic effects slowing motor neuron loss and NMJ abnormalities (Lesbordes et al., 2003).

Severe SMA mice have reduced levels of liver-derived insulin-like growth factor 1 (IGF-1) and IGF-binding protein acid labile subunit (IGFALS) mRNA, encoding a hepatic protein that stabilizes IGF-1 (Hua et al., 2011). As IGF-1 is released in response to circulating growth hormone released from the pituitary and required for normal growth and health maintenance, low levels of IGF-1 could explain the small size and growth retardation of SMA mice (Hua et al., 2011). Peripheral restoration of SMN is capable of increasing IGF-1 levels to normal (Hua et al., 2011) and conversely systemic administration of AAV1-IGF1 is capable of increasing SMN levels and improving disease manifestations of Taiwanese SMA mice (Tsai et al., 2014).

1.6.2.3 Neuroprotective agents

The neuroprotective agent riluzole is the only approved treatment for ALS where it has been shown to modestly increase survival by 2-3 months in patients (Bellingham, 2011). The precise mechanisms of riluzole’s protective action remain controversial and incompletely understood although it is generally accepted that a block of tetrodotoxin sensitive voltage-dependent sodium current and a reduction in glutamate signalling contribute to its clinical efficiency (Bellingham, 2011). In a SMA mouse
model which carries a neuronal specific Smn exon 7 deletion, riluzole treatment modestly improved survival from 33 to 38 days and reduced the number of abnormal NMJs with neurofilament accumulation although weight and motor performance remained unaffected (Haddad et al., 2003). In a small clinical trial in 10 Type I SMA children riluzole was well tolerated although no patients showed any change in motor abilities (Russman et al., 2003). Three of the treated infants followed an unusual course of disease progression living beyond the expected lifespan. However, due to the low sample size larger future trials would be required to assess any mitigating effects (Russman et al., 2003). The pharmokinetic properties of riluzole have more recently been investigated in 14 SMA patients in view of selecting a dose for a future larger clinical trial (Abbara et al., 2011).

Gabapentin, a structural analogue of the inhibitory neurotransmitter GABA, is an important drug in the management of seizure disorders and neuropathic pain (Rose and Kam, 2002). Gabapentin potentiates inhibitory GABAergic pathways (although it does not bind GABA_A and GABA_B receptors) and blocks L-type voltage-gated calcium channels leading to reduced excitatory amino acid neurotransmission (Rose and Kam, 2002). The first clinical trial of gabapentin in 84 Type II or III SMA patients showed no difference in muscle strength and overall functional outcomes between treated and placebo patients (Miller et al., 2001). A later trial of 120 Type II-III SMA patients, however, indicated improvements in arm and leg function with gabapentin although there was no change in disability as measured by functional timed tests (Merlini et al., 2003).

As motor neuron degeneration in SMA is in part mediated by apoptosis, anti-apoptotic agents may be of benefit to patients. In particular, the apoptosis regulator Bcl-2 family of proteins that govern mitochondrial permeabilization and can be either pro-apoptotic (e.g. Bax) or anti-apoptotic (e.g. Bcl2 and Bcl-xl) could be of particular interest. Reduced levels of the anti-apoptotic Bcl2 and Bcl-xl proteins have been noted in Type I SMA patient motor neurons during foetal development (Soler-Botija et al., 2003). Overexpression of Bcl-xl is able to prevent neurite degeneration and cell death in embryonic mouse motor neuron cultures with Smn knockdown.
In a Type III SMA mouse model, Bcl-xl overexpression under control of an early pan-neuronal promoter was able to protect against motor neuron loss, improve motor function and increase lifespan without increasing SMN levels (Tsai et al., 2008b); however, no increase in survival was seen in the Type I Taiwanese mouse model (Tsai et al., 2008b). In addition to the reduction of anti-apoptotic proteins an increase in pro-apoptotic Bax protein has also been identified in the spinal cord of Type I-III SMA mice (Tsai et al., 2006). Knockout of Bax in a Type II SMA mouse slowed phenotype progression and improved survival indicating that inhibition of Bax mediated apoptosis can ameliorate SMA disease severity (Tsai et al., 2006).

1.6.2.4 Myotrophic treatments

Therapies that promote muscle maintenance and growth have been proposed as a therapy for SMA. One of the most well studied targets is myostatin, a member of the transforming growth factor beta (TGF-β) family and a negative regulator of muscle skeletal muscle mass, with its genetic ablation causing generalized muscular hypertrophy in mice (Grobet et al., 2003). Myostatin mutations associated with increased muscle size have also been reported in children (Schuelke et al., 2004). Delivery of recombinant follistatin, a protein that binds myostatin and inhibits its activity, to SMNΔ7 mice was associated with improved muscle weight, motor function, reduced motor neuron loss and increased survival without increased SMN levels (Rose et al., 2009). Transgenic inactivation of myostatin however did not improve SMNΔ7 mouse survival, motor function or neuropathology and only minimally improved muscle weight (Rindt et al., 2012). Transgenic overexpression of follistatin in skeletal muscle of SMNΔ7 mice also did not improve muscle mass or the disease phenotype suggesting that recombinant follistatin may have off target effects or effect on organs other than skeletal muscle that could explain the previous beneficial findings (Rindt et al., 2012).

Muscle atrophy was found to be decreased in Type II mice exposed to a regular exercise regime (Grondard et al., 2005). Running in a wheel with a controlled speed for 20 minutes per day also prolonged survival and limited the extent of motor
neuron loss by preventing apoptosis (Grondard et al., 2005). Physical exercise also partially corrects cardiac muscle atrophy and improves cardiac function in these mice (Biondi et al., 2012). Clinical trials of physical exercise are currently underway in SMA children, including assisted standing in Type I infants and aerobic and/or strength training in Type II-III patients (Treat-Nmd, 2011). Stationary cycle training for 30 minute sessions 2-4 times per week for 12 weeks resulted in increased lung capacity but failed to improve muscle function in 6 SMA Type III adult patients (Madsen et al., 2014). Significant exercise-induced fatigue was induced by cycle training in these patients which caused an increase need for sleep and training protocol modification (Madsen et al., 2014). A pilot study of progressive strength training of 14 bilateral proximal muscles 3 times weekly for 12 weeks was associated with no adverse advents and a trend of improved motor strength and performance in 9 Type II-III patients (Lewelt et al., 2015). Daily whole body vibration training combined with standing exercises for 8 weeks showed a trend in muscle function, strength and flexibility in 8 ambulatory SMA children including a significant increase in timed walking distance after 8 weeks of treatment (Vry et al., 2014) indicating that strength training may be better than aerobic training for future clinical trials.

1.6.2.5 The ubiquitin proteasome system

As discussed in detail previously, SMN degradation is predominately regulated by the UPS. Alterations of the UPS have been observed in SMA mouse models where targeting UPS pathways has resulted in beneficial results. Inhibition of the proteasome in SMNΔ7 mice using bortezomib treatment doubled SMN protein levels in the liver, kidney and muscle (Kwon et al., 2011a). SMN levels in the spinal cord and brain remained unaffected probably due to bortezomib’s low CNS penetrance (Kwon et al., 2011a). This increase in peripheral SMN levels was associated with an improvement of motor function although survival and motor neuron counts were unaffected (Kwon et al., 2011a). A combinatory approach to increase SMN levels using the HDACi TSA and prevent SMN degradation by using bortezomib resulted in a synergistic improvement in the phenotype of SMNΔ7 mice (Kwon et al., 2011a). TSA plus bortezomib treatment resulted in a more improved muscle fibre,
NMJ and motor neuron phenotype compared to either treatment alone (Kwon et al., 2011a). A synergist effect was also seen with lifespan which increased by 6 days with combinatory treatment compared to 3 days with TSA alone (Kwon et al., 2011a).

An exciting novel mechanistic link between SMN and maintenance of ubiquitin homeostasis has also recently been discovered (Wishart et al., 2014). As discussed previously, perturbations in UPS pathways, in particular decreased levels of Uba1, lead to accumulation of β-catenin in the neuromuscular system of SMA mice (Wishart et al., 2014). Impressively, inhibition of β-catenin signaling using the plant based flavonoid compound quercetin rescued the motor neuron defects in Uba1 inhibitor treated zebrafish and reduced neuromuscular pathology in zebrafish and Drosophila SMA models (Wishart et al., 2014). β-catenin inhibition was also able to ameliorate neuromuscular pathology in severe Taiwanese SMA mice whereby quercetin robustly increased motor performance, reduced motor neuron loss, increased muscle fibre diameter and improved NMJ morphology to levels comparable to controls (Wishart et al., 2014). Targeting, β-catenin signalling, or other upstream ubiquitin pathways, therefore represents an attractive therapeutic strategy for promoting neuromuscular stability and function in SMA. Together this report combined with previous studies demonstrating the importance of SMN ubiquitin-dependent degradation and stability show that targeting the UPS is an attractive therapeutic target for SMA and promising avenue for future work to further understand mechanisms underlying SMA pathogenesis.
1.7 Summary

In this introduction I have reviewed the currently published literature regarding the clinical phenotype, genetics, animal models and underlying molecular pathogenesis of SMA. I have also discussed the limitations of traditional SMN dependent therapeutic strategies and the importance of exploring non-SMN dependent targets. Of these non-canonical pathways, the UPS is an exciting new area of SMA research with recently identified alterations in the UPS (including reduced levels of UBA1 and increased levels of UCHL1 and β-catenin) providing promising new targets for therapy development.
1.8 Aims

In this thesis I will therefore further examine the role of the UPS in a range of SMA models and explore targeting of the UPS to develop novel therapeutic strategies in SMA mice. In particular the following aims will be addressed:

1) Alterations in the UPS have been described in the neuromuscular system of ‘Taiwanese’ and ‘US’ SMA mice. I will aim to establish whether UPS perturbations are present in other organ systems of the ‘Taiwanese’ SMA mice and in other SMA models including $Smn^{2B/-}$ mice, zebrafish and patient derived iPSC motor neurons.

2) Levels of β-catenin are increased in the neuromuscular system of SMA mice and as inhibition of β-catenin using the flavonoid compound quercetin was able to ameliorate neuromuscular pathology I will aim to assess if other β-catenin inhibitors also provide similar or improved enhancements in the motor performance of SMA mice.

3) As levels of the deubiquitinating enzyme UCHL1 are robustly increased in SMA patient cells and mouse models with UCHL1 inhibition increasing SMN levels in vitro, I will therefore aim to establish whether pharmacological inhibition of the deubiquitinating enzyme UCHL1 can improve, survival, motor symptoms and neuromuscular pathology in a mouse model of SMA.

4) Given that Uba1 levels are significantly reduced in SMA mice and that genetic or pharmacological suppression of Uba1 is sufficient to recapitulate SMA-like neuromuscular pathology in zebrafish I will aim to assess if increasing UBA1 levels using gene therapy is an effective therapeutic strategy in SMA mice.
Chapter 2. Materials and methods

2.1 Ethics statement

Experimental procedures throughout were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. All animal experiments were approved by a University of Edinburgh internal ethics committee and were performed under Home Office regulations under project licence number 60/4569 and personal licence number 60/13691.

2.2 In vitro experiments

2.2.1 SMA patient and control iPSC derived motor neurons

iPSCs derived motor neurons were made by Julien Come and Dr. Cecile Martinat, I-Stem Institute, France by reprogramming Coriell Biorepository patient fibroblasts as previously described (Maury et al., 2015). 8 independent clones of control iPSC motor neurons were generated from fibroblast line GM03814 and 9 independent SMA clones generated from fibroblast lines GM03813 or GM00232. Western blot analysis was performed on cells that were harvested at 1 day post-differentiation.

2.2.2 SMA patient and control primary fibroblast cell culture

Primary fibroblasts were obtained from the laboratory of Prof. Brunhilde Wirth, Insitute of Human Genetics, University of Cologne, Germany. Cells were previously generated from skin biopsies from three SMA Type I patients with homozygous deletion of SMN1: ML-16, ML-17, ML-83 and three age matched unaffected controls: ML-24, ML-32, ML-44. Cells were maintained in high glucose Dulbecco’s modified Eagle medium (DMEM) (Gibco, Invitrogen) supplemented with 10% heat inactivated foetal bovine serum (HyClone, Thermo Scientific) and 100U/ml penicillin and 100mg/ml streptomycin (Gibo, Invitrogen). Fibroblasts were expanded and harvested for protein on or below passage 8.


### 2.2.3 Uba1 pharmacological inhibition on primary hippocampal neuronal cells

Western blot experiments were performed on cells previously treated by Dr. Chantal Mutsaers. E17 rat hippocampal neurons were cultured in Basal Medium Eagle (BME) serum free medium complemented with 1.6% of a 32.5% glucose solution, 1% Sodium Pyruvate 100 nM solution, 1% N-2 supplement and 5% B27 supplement. Cells were plated in a12-well plate and kept in 1ml of media. The cells were treated with 50uM UBEI-41, a cell-permeable ubiquitin E1 inhibitor with an IC50 ~ 5 µM (Biogenova) dissolved in DMSO or DMSO only vehicle control for 2 hrs before the cells were harvested for protein.

### 2.3 Mouse and zebrafish models of SMA

#### 2.3.1 Taiwanese SMA mouse model and colony maintenance

The ‘Taiwanese’ mouse model of severe SMA (Smn\(^{+/−}\); SMN2\(^{+/0}\)) was used throughout (Hsieh-Li et al., 2000) using the breeding strategy developed by (Riessland et al., 2010). The SMA Taiwanese mouse line (referred to as TTG) were generated by breeding programme of crossing THET Smn heterozygous mice (Smn\(^{+/−}\)) with THOM mice which are Smn null (HRTP knock-out of exon 7) and carry 4 copies of the human SMN2 transgene (Smn\(^{+/−}\); SMN2\(^{tg/tg}\)). The Smn homozygous (Smn\(^{+/+}\)) and Smn heterozygous (Smn\(^{+/−}\)) members of the THET colony are phenotypically normal whilst the mice in the THOM colony show a mild phenotype of developing necrotic ears and tails. In the resulting TTG colony, the Smn heterozygous mice with 2 copies of the SMN2 transgene (Smn\(^{+/−}\); SMN2\(^{tg/0}\)) are phenotypically normal and are referred to as control littermates whilst the Smn homozygous mice with 2 copies of SMN2 (Smn\(^{+/−}\); SMN2\(^{tg/0}\)) are referred to as SMA mice. SMA mice have a lifespan of around 9 to 11 days and display a progressive phenotype of reduced body weight, muscle wasting, motor neuron degeneration and systemic organ pathology. Taiwanese SMA mice on a congenic FVB background were originally obtained from Jackson Laboratories (strain name: FVB.Cg-Tg(SMN2)2Hung Smn\(^{1m1Hung}\)/J, stock number: 005058) and were maintained as
breeding colonies in the animal care facilities in Edinburgh under standard specific pathogen free conditions.

### 2.3.1.1 Genotyping

For DNA extraction, ear punches or tail tips were added to 500ul lysis buffer (100mM Tris pH8, 200mM NaCl, 5mM EDTA pH8, 0.2% SDS in ddH$_2$O) containing 2.5ul Proteinase K (ThermoScientific) and left to digest overnight at 55°C. Samples were then mixed and spun for 15min at 14,000 rpm. DNA was precipitated by pouring the resulting supernatant into 500ul isopropanol and mixed by inversion. The DNA pellet was then washed twice with 70% ethanol for 30 secs at 14,000 rpm. Following drying, the DNA was then left to solubilise in either 50ul or 200ul deionised water for ear punches or tail tips respectively.

PCR was performed using the following primers (all Sigma Aldrich). Product size in base pairs is indicated in brackets:

- **Smn**
  - Forward: ATAACACCACCACTCTTACTC
  - Reverse 1: GTAGCCGTGATGCCATTGTCA (1050 bp)
  - Reverse 2: AGCCTGAAGAAGCAGATCAGC (950 bp)

- **SMN2**
  - Forward: CGAATCACTTGAGGGCAGGAGTTTG
  - Reverse: AACTGGTGGACATGGCTGTTCATTG (450 bp)

PCR master mixes were as follows (all regents PCR Master Mix, Promega):

- **Smn**
  - 3ul 5x buffer green
  - 0.9ul 25M MgCl
  - 0.15ul dNTPs
  - 0.2ul Taq DNA polymerase
  - 1.5ul 10uM Forward *Smn* primer
  - 1ul 10uM Reverse 1 *Smn* primer
  - 1ul 10uM Reverse 2 *Smn* primer
  - 6.25ul ddH$_2$O

- **SMN2**
  - 3ul 5x buffer green
  - 0.9ul 25M MgCl
  - 0.15ul dNTPs
  - 0.2ul Taq DNA polymerase
  - 1.5ul 10uM Forward *SMN2* primer
  - 1.5ul 10uM Reverse *SMN2* primer
  - 8.25ul ddH$_2$O
Amplification was performed using a thermocycler using the following program:

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<thead>
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<th>Time</th>
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</thead>
<tbody>
<tr>
<td>1</td>
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<td>3 min</td>
</tr>
<tr>
<td>2</td>
<td>94°C</td>
<td>30 secs</td>
</tr>
<tr>
<td>3</td>
<td>59°C</td>
<td>30 secs</td>
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<tr>
<td>4</td>
<td>68°C</td>
<td>60 secs</td>
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<tr>
<td>5</td>
<td>Repeat steps 2 – 4 (x 35)</td>
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<tr>
<td>6</td>
<td>68°C</td>
<td>5 min</td>
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<tr>
<td>7</td>
<td>4°C</td>
<td>hold indefinitely</td>
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</table>

PCR products were then separated by gel electrophoresis. 1% agarose gels were used made and ran with TAE (40mM Tris base, 40mM acetate, 1mM EDTA in ddH₂O) containing SYBR Safe DNA gel stain (Life Technologies). Example genotypes are shown in Figure 2-1.

![Figure 2-1. Example Smn and SMN2 genotyping of the THET, THOM and TTG mouse colonies](image)

Figure 2-1. Example Smn and SMN2 genotyping of the THET, THOM and TTG mouse colonies Lane order: 1. 100bp DNA ladder, 2. THET heterozygote (Smn⁺⁻), 3. THET homozygote (Smn⁺⁺), 4. THOM (Smn⁻⁻; SMN2⁰⁰), 5. TTG heterozygote (Smn⁻⁻; SMN2⁰⁰), 6. TTG homozygote (Smn⁻⁻; SMN2⁰⁰).
2.3.2 Smn\textsuperscript{2B/-} mouse tissue

Tissue dissected from Smn\textsuperscript{2B/-} SMA mice which were provided by Dr. Lyndsay Murray. Smn\textsuperscript{2B/-} mice on a mixed FVB-C57BL6 background were taken at P18 where tissue was dissected quickly on dry ice and stored at -80\degree C until further use. Genotyping was performed by Natalie Courtney. Smn\textsuperscript{+/+} littermates were used as wild type controls.

2.3.3 SMA zebrafish tissue

Western blot experiments were performed on a zebrafish model of SMA (Mcwhorter et al., 2003) generated by Dr. Penelope Boyd. Embryos of the Tg(hb9:GFP) transgenic line (Flanagan-Stee et al., 2005) were microinjected with 4nM or 6nM smn morpholino (smn: 5’-CGACATCTTCTGCACCATTGGC-3’; Gene Tools, LLC) at the one cell stage. At 48 hrs post fertilization, embryos were manually dechorionated using fine forceps and pooled into batches of 30 fish, with three replicate batches per experimental group. In preparation of tissue for western blot, yolk sacs were removed by mechanical stress (Link et al., 2006). Following pipetting with a narrow tip in Ginsburg fish ringer buffer (55 mM NaCl, 1.8 mM KCl, 1.25 mM NaHCO\textsubscript{3}) the fish were shaken at 5 min at 1100 rpm. A wash buffer (110 mM NaCl, 3.5 mM KCl, 2.7 mM CaCl\textsubscript{2}, 10 mM Tris/Cl pH8.5) was then applied followed by shaking 2 min at 1100 rpm, and repeated before pelleting the fish, freezing on dry ice and storing at -80\degree C.

2.4 \textit{In vivo} drug trials in Taiwanese SMA mice

It is important to note that due to inbreeding and genetic drift, the phenotype of the SMA mice worsened overtime (e.g. mean survival 11 days in February 2012 vs mean survival of 9 days in August 2014). All drug trials were therefore completed independently of each other and data not compared against other previous data sets.
2.4.1 Beta-catenin inhibitors

Compounds with reported beta-catenin inhibition (aspirin, indomethacin and iCRT-14) were trailed to assess their therapeutic potential in SMA mice. Aspirin (100mg/Kg or 25mg/Kg; Sigma-Aldrich), indomethacin (4mg/Kg; Sigma-Aldrich) and iCRT-14 (20mg/Kg; Tocris Bioscience) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) administered by intraperitoneal (IP) injection once daily from postnatal day one (P1) using a 30 gauge needle. These doses were based on the published literature of previously been delivered safely to mice with beneficial effects of beta-catenin inhibition for aspirin (Stark et al., 2007), indomethacin (Heidel et al., 2012, Shenoy et al., 2012) and iCRT-14 (Gonsalves et al., 2011). Drug solutions were made up to minimise the injection volume. The same volume of DMSO was administered by IP injection as a vehicle-only control. TTG litters were randomly assigned to either treatment of vehicle groups. Each pup was weighed, numbered with marker pen and after injection, rolled in bedding and returned to the cage with their parents.

2.4.2 Uch11 inhibition

LDN-57444 (Calbiochem) is an isatin O-acyl oxime compound that acts as a potent, reversible, competitive, and active site-directed inhibitor of UCHL1 ($K_i = 0.40 \mu M$; $IC_{50} = 0.88 \mu M$). LDN-57444 was dissolved in DMSO and administered 0.5 mg/kg, twice daily to TTG pups via IP injection from the day of birth. This dose was based on previous studies reporting this concentration to be effective in vivo (Gong et al., 2006, Cartier et al., 2012). Tissue from P9 LDN-57444 treated control and SMA mice was taken for neuromuscular pathology analysis.

2.4.3 AAV9-UBA1 gene therapy

AAV9 vectors containing a full-length human UBA1 open reading frame (ORF) cDNA construct were made by Eva Karyka in the laboratory of Prof. Mimoun Azzouz at the University of Sheffield. AAV9-GFP control vectors were also provided by Prof. Mimoun Azzouz. All gene therapy injections in TTG or CD1 wild-
type mice were conducted under chilled anaesthesia using a 33 gauge needle and Hamilton syringe. AAV9-UBA1 was administered at concentration of 2.4xE+11 vg and 1.4xE+11 vg for AAV9-GFP. IP injections were delivered at a volume of 30ul whilst for IV delivery; 10ul was given via the facial vein. Visualisation of facial veins for IV injection was aided by using a Wee Sight Transilluminator LED light (Philips). Successful IV injection was noted by blanching of the vein during injection. Bleeding at the injection site was minimised by applying pressure with a tissue. Pups were hand held until their temperature and movement returned to normal then placed back in their cage. Following facial vein injections, maternal neglect was noticed in the TTG colony. For this reason, TTG pups undergoing subsequent IV injections were fostered on to CD1 mothers. Uninjected TTG pups with CD1 fostering were used as controls. To analyse the temporal bio-distribution of AAV9 gene therapy, CD1 mice which had received AAV9-GFP injection at P1 were culled at P3, P4, P5, P6, P7, P8, P30 and P60 for tissue analysis. TTG mice with AAV9-UBA1 delivered IP were culled at P8 to analyse efficacy of AAV9-UBA1 gene therapy at increasing UBA1 levels in vivo. In subsequent IV delivery, TTG mice were culled at P7 for analysis of protein expression and P9 for analysis of neuromuscular pathology.

2.4.4 Weight, survival and health monitoring

Pups undergoing drug or gene therapy trials were checked, weighed and monitored for survival daily. If the pups appeared very unhealthy (based on an evaluation of their established and robust clinical phenotype [e.g. reduced activity and mobility, tremor, diarrhoea, delayed development] alongside their performance on the behavioural righting tests) and/or experienced weight loss of 20% from the peak body weight of that animal, persisting for 72 hrs they were culled for humane reasons. If taken for tissue analysis mice were killed by Schedule 1 procedures.

2.4.5 Motor performance testing

To assess the impact of drug or gene therapy trials on motor performance a righting test was performed. The righting reflex is a commonly used simple assay to assess
motor ability in neonatal mice and was performed by placing a mouse on its back on a flat surface and measuring the time taken to turn over onto its paws (Passini et al., 2010). If a mouse did not respond within 60 secs the test was terminated.

2.4.6 Evaluation of blood chemistry

To further evaluate the safety of AAV9-UBA1 gene therapy and an analysis of blood cell counts and serum chemistry was conducted. Under terminal anaesthesia, blood was collected via cardiac puncture from 1 month control TTG mice that had received an AAV9-UBA1 injection at P1. 1 month old TTG mice that had received no treatment or AAV9-GFP injections were used as controls. Blood was collected into heparin coated tubes, kept on ice and immediately taken to the University of Edinburgh Veterinary Pathology Unit where a ‘small animal health check profile’ was performed.

2.5 Neuromuscular pathology analysis

2.5.1 NMJ analysis

NMJ axonal input quantification for AAV9-UBA1 treated mice was performed by Dr. Ross Jones. Cranial muscles were dissected as previously described (Murray et al., 2010a) and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in 0.1M phosphate-buffered saline (PBS) for 10 min on a rocking platform at room temperature. Following washing in PBS the levator auris longus (LAL) was dissected out and connective tissue removed. LAL muscles were then washed in 2% Triton X-100 (Sigma-Aldrich) in PBS for 30 min and incubated in blocking solution (4% bovine serum albumin [BSA, Sigma-Aldrich] and 1% Triton X-100 in PBS) for 30 min. Muscles were incubated in mouse anti-2H3 neurofilament (1:200, Developmental Studies Hybridoma Bank [DSHB]) and mouse anti SV2 synaptic vesicle (1:100, DSHB) primary antibodies in blocking solution overnight at 4°C. Following 3 x 20 min PBS washes, muscles were incubated in TRITC-conjugated alpha-bungarotoxin solution (1:800, Biotium) made in PBS for 10 mins at room temperature. Muscles were then placed in DyLight 488 donkey anti-mouse secondary
antibody (1:100, Jackson) for 2 hrs at room temperature. Following 3 x 10 min washes, muscles were mounted and on slides and cover-slipped with Mowoil (Polyscience) mounting media with DABCO [1,4-diazobicyc[2.2.2]-octane, Sigma-Aldrich] to 2.5% w/v to reduce fading of fluorophores. NMJ analysis was performed using a Nikon Eclipse 50i upright epifluorescent microscope at x60 objective. For NMJ analysis the rostral and caudal bands were analysed separately with approximately 50 NMJs per band chosen at random. To evaluate the number of neural inputs, the number of axons converging on a single motor endplate was counted. Example images were taken using a Nikon A1R confocal system combined with Ti:E inverted microscope (Objective: x60 Plan Apo OIL, NA=1.4).

2.5.2 Muscle fibre diameter measurements

LAL and transversus abdominis (TVA) were whole mounted on to slides and cover-slipped with Mowoil mounting media. Phase contrast images muscles were taken using an inverted Olympus 1X71 microscope at 20x objective and images taken using a digital CCD camera (Hamamatsu C4742-95) and OpenLab (Improvision) image capture software. Images were taken around the edge of dissected muscles were individual fibres could be ascertained. Muscle fibre diameter measurements were made using ImageJ software (Schneider et al., 2012).

2.5.3 Motor neuron cell body counts

The isolated vertebral column was removed and fixed in 4% paraformaldehyde in PBS overnight at 4°C. Spinal cords were removed from vertebral columns and placed in 30% sucrose solution in PBS overnight for cryoprotection. The lumbar enlargement was dissected and placed in plastic moulds with optimal cutting temperature compound (OCT, Cell-Path), frozen on dry ice and stored at -80°C. 25um sections were cut using a cryostat (Leica, CM3050S) and every 4th section collected onto Superfrost Plus slides (Thermo Scientific) and stored at -20°C. For Nissl staining, slides were brought to room temperature, rinsed in water, dehydrated up through graded alcohols (70%, 90% and 100% ethanol) before being paced in xylene for 10 min. Sections were then rehydrated back down the graded alcohols and
water before being stained in 0.2% Cresyl Fast Violet containing 1% acetic acid for 7 min. Following dehydration up graded alcohol solutions and xylene, slides were coverslipped using DPX mounting media (Fisher-Scientific). Phase contrast images of the ventral horn were taken using an inverted Olympus 1X71 microscope at 4x objective and images taken using a digital CCD camera (Hamamatsu C4742-95) and OpenLab (Improvision) image capture software. Motor neuron cell body numbers were analysed by counting the number large darkly stained anterior horn cells per ventral horn.

2.6 **UCHL1, UBA1 and GFP immunohistochemistry**

Tissue was dissected and fixed in 4% paraformaldehyde in PBS for 4 hrs at 4°C before being transferred in to 30% sucrose solution overnight at 4°C for cryoprotection. Tissue was then embedded in OCT and stored at -80°C. Using a cryostat, heart and liver were sectioned at 20um whilst spinal cord, brain and muscle was sectioned at 25-30um and collected on to Superfrost Plus slides (Thermo Scientific). Using a Shandon Sequenza Immunostaining Centre (Thermo-Scientific) sections were permeabilised in 0.3% Triton X-100 in PBS then blocking solution (4% BSA, 0.3% Triton X-100 in PBS) for 30 min at room temperature before overnight incubation with primary antibody solution at 4°C. The following antibodies were used: rabbit anti-UCHL1 (1:500, Novus Biologicals, NB300-676), rabbit anti-GFP (1:500, Abcam, ab290), mouse anti-UBA1 (1:200, Sigma-Aldrich, E3125). After 3 x 10 min washes in PBS, sections were incubated with secondary antibody solution for 2 hrs at room temperature before being mounted on to slides. The following secondary antibodies were used: swine anti-rabbit IgG TRITC (1:100, DAKO, R0156), Alexa Flour 488 donkey anti-mouse IgG (1:500, Life Technologies, A21202), donkey anti-mouse IgG (1:500, Life Technologies, A21202), Alexa Flour 555 donkey anti-mouse IgG Alexa Flour (1:500, Life Technologies, A31570). Sections were sometimes counterstained with Neurotrace 435/455 blue fluorescent Nissl stain (1:100, Life Technologies, N21479) or DAPI nuclei stain (1:1,000, Life Technologies, D1306) for 10 mins. Secondary antibody only control sections were treated in an identical manner except for the exclusion of primary antibody. Images
were taken using an inverted Olympus 1X71 microscope with digital CCD camera (Hamamatsu C4742-95) and OpenLab (Improvision) image capture software or a Nikon A1R confocal system combined with Ti:E inverted microscope.

2.7 Quantitative fluorescent western blot analysis

2.7.1 Protein extraction and quantification

Tissue for western blot analysis was tissue was quickly dissected, placed on dry ice and stored at -80°C. To extract protein, tissue and cells were homogenised on ice using a motorised disposable pestle mixer (VWR) in radioimmunoprecipitation assay (RIPA) buffer (ThermoScientific) with 1% protease inhibitor cocktail (Sigma). The protein concentration was then determined by bicinchoninic acid (BCA) assay (ThermoScientific).

2.7.2 Polyacrylamide gel electrophoresis

Extracted protein was diluted to a final concentration of 40ug/well in water and NuPAGE LDS sample buffer (Life Technologies) and heated to 95°C for 5 min. Protein samples were then separated by polyacrylamide gel electrophoresis for 40 min at 180 volts on 4–20% precast NuPage 4–12% BisTris gradient gels (Life Technologies) and ran alongside a Novex sharp pre-stained protein standard (Life Technologies, LC5800). Gel embedded proteins were then transferred to polyvinylidene difluoride (PVDF) membranes by the iBlot 7 minute semi-dry blotting system (Life Technologies).

2.7.3 Protein detection and western blot analysis

Total protein levels were determined by incubation of each membrane in a solution of Ponceau S (0.2% Ponceau, 30% acaetic acid) for 20 min. PVDF membranes were then placed in blocking solution (LI-COR Biosciences) for 30 min at room temperature incubated in primary antibody solution overnight. The following primary antibodies were used: rabbit anti-UCHL1 (1:2,000, Novus Biologicals,
Chapter 2. Materials and methods

2.8 Real-time reverse transcription PCR (RT-qPCR)

2.8.1 RNA extraction

RT-qPCR experiments were performed with the help of Dr. Gillian Hunter. Extraction of total RNA was achieved by using RNAeasy Mini kit (Qiagen). Briefly, RLT buffer containing 1% beta-mercaptoethanol was added to tissue samples that had been stored at -80°C. Tissue was homogenised in RLT buffer and lysate pipetted onto QIAshredder spin column (Qiagen) and spun at 14000 rpm for 2 min. 1 x volume of 70% ethanol was added to lysate and transferred to RNAeasy spin columns and spun at 10000 rpm for 30secs. Discarding the flow through 350ul RW1 buffer was added to the columns and spun for 10000 rpm for 30secs. 70ul RDD
buffer mixed with 10ul DNaseI (Qiagen) was added onto column and left at room temperature for 15min. After, 350ul RW1 buffer was added and the column spun at 10000 rpm for 30secs. Discarding the flow through this step was repeated twice with 500ul RPE buffer. The column was then spun for 2 min to get rid of any remaining buffer. RNA was collected by transferring the column to a new 1.5ml tube and eluting with 80ul RNAase free water. Eluted RNA was then aliquoted and stored at -80°C until further use.

2.8.2 RNA integrity and quantification

RNA concentration was determined by Nanodrop 2000 spectrophotometer (Thermo Scientific) analysis. The A260/230 and A260/280 ratios were noted to determine RNA purity. Quantified samples had A260/280 ratios ranging from 2.06-2.09 and A260/203 ratios from 1.83-2.08. To determine integrity, 1.5ul RNA mixed with 1ul loading dye (Promega) was run on a 1% agarose gel at 120V for 30min. The presence of two strong 28S and 18S rRNA bands in a ratio of 2:1 ratio 28S:18S allowed for the visualisation of intact RNA.

2.8.3 RT-PCR

cDNA was made from RNA using a High Capacity cDNA reverse transcription kit (Life Technologies). RNA was diluted to a concentration of 1.5ug and made up to a 10ul volume per reaction in RNA free water. For each sample two reactions were performed: one with reverse transcriptase (RT), the other without (Neg RT control).

**RT positive**
- 2ul 10x RT buffer
- 0.8ul 25x dNTP
- 2ul 10x RT random primer
- 0.1ul Reverse transcriptase
- 4.2ul Nuclease free water

**Neg RT control**
- 2ul 10x RT buffer
- 0.8ul 25x dNTP
- 2ul 10x RT random primer
- 0ul Reverse transcriptase
- 5.2ul 10uM Nuclease free water

10ul RT-PCR mix was added to 10ul RNA and RT-PCR performed using a
thermocycler using the following program:

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<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
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</thead>
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<tr>
<td>2</td>
<td>37°C</td>
<td>2 hrs</td>
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<td>85°C</td>
<td>5 min</td>
</tr>
<tr>
<td>4</td>
<td>4°C</td>
<td>hold indefinitely</td>
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</table>

### 2.8.4 qPCR primer design

To design primers specific for mouse and human *Uba1*, the National Center for Biotechnology Information (NCBI) database was used to identify the nucleotide sequence for coding sequence of the genes. Both transcript variant 1 and 2 were noted for mouse (NM_009457.4 and NM_001136085.2) and human (NM_003334.3 and NM_153280.2) *Uba1* genes. FASTA nucleotide sequences were aligned using the multiple sequence alignment program ClustalW2. Primers were designed to span exon-exon boundaries identified by the Ensemble genome browser and were chosen based on regions that were either 1) unique to human *UBA1* 2) unique to mouse *Uba1* or 3) in conserved regions in mouse and human. Primer sequences were checked for sequence specificity using the BLAST tool on NCBI. SMN primers were chosen from the published literature (Hua et al., 2010). Specificity of primers was tested and using PCR with products shown in Figures 6-8 and 6-14.

### 2.8.5 qPCR primer optimisation

To validate qPCR primers and ensure that they amplify correctly a temperature gradient and standard curve was initially performed for each primer pair. The optimal annealing temperature was determined by performing a thermal gradient of 55-65°C and assessing which temperature gave the lowest Ct values and strong single melting curve peak. Using this temperature a standard curve was performed using serial dilutions (1.5 dilution factor) of cDNA and primer pairs considered acceptable if the efficiency was between 90-110%. If the efficiency of the reaction was outside that range further optimisation was performed by varying the primer concentration.
The following optimised primers were used for subsequent experiments:

**Mouse Uba1:**
- Forward: CCTACATGACCAAGGAACTA
- Reverse: AGCTGTTGAGTTCCAGCAAGT
- Size: 122bp  
- Temp: 64.5°C  
- Primer volume: 0.4μl  
- Efficiency: 95%

**Human UBA1:**
- Forward: TCTGTGCTCAGCATGGCG
- Reverse: ATGAGGTCCTCGTCAGGTTA
- Size: 133bp  
- Temp: 63.3°C  
- Primer volume: 0.3μl  
- Efficiency: 110%

**Mouse Uba1 and Human UBA1:**
- Forward: TGTCCAAGAAACGTCGCG
- Reverse: CTGCTCTATGTCTGCTTCACT
- Size: 140bp  
- Temp: 64.5°C  
- Primer volume: 0.4μl  
- Efficiency: 104%

**FL-SMN:**
- Forward: GCTGATGCTTTGGGAAGTATGTTA
- Reverse: CACCTTCCTTTTTTGGATTTTGTC
- Size: 98bp  
- Temp: 64.5°C  
- Primer volume: 0.75μl  
- Efficiency: 108%

**Δ7-SMN:**
- Forward: TGGACCACCAATAATTCCCC
- Reverse: ATGCCAGCATTTCCATATAATAGCC
- Size: 137bp  
- Temp: 64.5°C  
- Primer volume: 0.4μl  
- Efficiency: 100%

**GAPDH:**
- Forward: CGTCCCGTGAGACAAAATGGT
- Reverse: GAATTGCGGTGAGGTGGAGT
- Size: 177bp  
- Temp: 57°C  
- Primer volume: 0.4μl  
- Efficiency: 94%

**OAZ1:**
- Forward: ATCCTCAACAGCCACTGCTT
- Reverse: CGGACCCAGGTACTACAGC
- Size: 162bp  
- Temp: 57°C  
- Primer volume: 0.4μl  
- Efficiency: 98%

**PPIA:**
- Forward: CGCGTCTCCTCGAGCTTGTG
- Reverse: TGAAAGTCCACCCCTGGGACAT
- Size: 145bp  
- Temp: 63.3°C  
- Primer volume: 0.4μl  
- Efficiency: 102%
For experiments, neat cDNA was diluted 1:3 in DNase free water. To minimise pipetting error cDNA was first diluted 1:6 before adding to 96 well plates. 14ul of KAPA SYBR FAST qPCR Master Mix (KAPA Biosystems) and primer mix were added to make a total volume of 20ul per reaction e.g.:

<table>
<thead>
<tr>
<th>cDNA</th>
<th>qPCR Master mix</th>
</tr>
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<tbody>
<tr>
<td>1ul DNA</td>
<td>10ul KAPA SYBR FAST qPCR master mix</td>
</tr>
<tr>
<td>5ul DNase free water</td>
<td>0.4ul Forward primer (10uM)(volume variable)</td>
</tr>
<tr>
<td></td>
<td>0.4ul Reverse primer (10uM) (volume variable)</td>
</tr>
<tr>
<td></td>
<td>3.2ul DNase free water (volume variable)</td>
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<tr>
<td><strong>Total: 6ul</strong></td>
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</table>

Three samples were used for each experimental condition with each sample was be amplified in triplicate. Negative RT controls and no template controls were performed for each sample and each primer pair. qPCR reactions were performed with a BioRad CFX Connect Optis module real time system thermocycler (Bio-Rad) using the following programme:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
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<tbody>
<tr>
<td>1</td>
<td>95°C</td>
<td>3 min</td>
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<tr>
<td>2</td>
<td>95°C</td>
<td>3 secs</td>
</tr>
<tr>
<td>3</td>
<td>Primer dependent</td>
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<td>15 secs (plate read)</td>
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<td>5</td>
<td>Repeat steps 2 – 4 (x 39)</td>
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<tr>
<td>6</td>
<td>95°C</td>
<td>10 secs</td>
</tr>
<tr>
<td>7</td>
<td>Melt curve 65-95°C</td>
<td>5 secs (plate read) in 0.5°C steps</td>
</tr>
</tbody>
</table>

qPCR analysis was performed using BioRad CFX manager 3.1 software. Normalising expression to housekeeping genes that were not changed under experimental conditions (GAPDH, OAZ1 and PPIA) a gene study analysis was performed to compare the expression of Uba1 and SMN expression in control and AAV9-UBA1 treated tissue. Samples with Cq values >35 cycles were considered as having no detectable expression.
2.9 Proteomic analysis

Samples for proteomic analysis were prepared by Sam Eaton. Heart tissue from P7 AAV-9 UBA1, AAV9-GFP and uninjected control mice (N=3) were homogenised in 500μl label free buffer (1.2g Tris(hydroxymethyl)aminomethane, 4g SDS in 100ml dH2O; pH 7.6) with Halt protease cocktail inhibitor (1:100, Life Technologies). Tissue was homogenised in M tubes using a gentleMACS Dissociator until macerated and the lysate settled on ice for 20 min. Lysate was transferred to protein Lo-Bind 1.5ml tubes (Eppendorf) and centrifuged at 14,000 rpm at 1°C for 20 min. Total protein quantification of the supernatant was carried using BCA assay (ThermoScientific) and verified using Coomasie Blue staining of PVDF membranes.

Label free proteomic analysis was undertaken at the FingerPrints Proteomic Facility, University of Dundee and *in silico* analysis performed with the help of Maica Llaverro and Dr. Tom Wishart. For label-free proteomic analysis, samples in each experimental group were pooled to minimise inter-sample variability and 3 liquid-chromatography mass-spectrometry (LC-MS) runs performed for each group. Raw data of peptide ions identified by LC-MS were imported to Progenesis QI Proteomics software (Nonlinear Dynamics) where ion intensity maps were aligned, a maximum detection ion charge limit of 5 was applied and retention time limits were set to eliminate analysis of ions eluted <16 min and >136 min which identified 106,306 ms/ms spectra. Filters were applied to identify ms/ms spectra with a significant ($P \leq 0.05$) 20% fold change between groups, those with a statistical power of above 80%, and those with a rank of ≥3 to identify only the most reliable spectra. This second filtering resulted in the identification of 23,665 ms/ms spectra which were exported and compared against the MASCOT mouse protein database (Matrix Science). The 8,406 peptide ions identified from MASCOT were reimported to Progenesis QI Proteomics software where 788 proteins in all three experimental groups were subsequently identified. A third filter was applied to find proteins with a significant ($P \leq 0.05$) 20% fold change between groups and which had been identified by ≥2 unique peptides resulting in 198 proteins changes in AAV9-UBA1 tissue compared to control. Of these proteins, 47 were also significantly changes in AAV-GFP treated
hearts and were filtered out as being a response to AAV9 injection. The remaining data set on 151 proteins was then analysed by Ingenuity Pathway Analysis software (Qiagen) to identify any protein pathway alterations in AAV9-UBA1 treated tissue.

2.10 Statistical analysis

Data were analysed using Microsoft Excel 2010 and GraphPad Prism 6 software. All data are reported as mean ± standard error mean (SEM). Individual statistical tests used are detailed in figure legends. Statistical significance was considered to be $P \leq 0.05$ for all analyses. Figures were created using Adobe Photoshop 6 software.
Chapter 3. UPS alterations in SMA models

3.1 Introduction

As discussed in detail in Chapter 1, alterations in the UPS have previously been described in the neuromuscular system of Taiwanese and US SMA mice (Wishart et al., 2014). In this chapter I aimed to establish whether similar UPS perturbations are present in other organ systems of the Taiwanese SMA mice, as well as in other SMA models, including Smn$^{2B/-}$ mice, zebrafish and patient derived iPSC motor neurons. Smn$^{2B/-}$ mice represent an intermediate SMA model in which Smn is modified to a humanised SMN2-like gene. Knock-in of the 2B allele (which contains point mutations in the Smn exon splice enhancer site that binds the splicing factor transformer 2 beta [Tra2B]) disturbs Smn splicing resulting in production of a Δ7Smn-like protein (Bowerman et al., 2009). Smn$^{2B/-}$ mice have a median survival of around 30 days and at P21 display motor neuron loss, muscle atrophy and NMJ defects (Bowerman et al., 2012a). In addition to neuromuscular pathology, alterations in metabolism have also been observed in Smn$^{2B/-}$ mice (Bowerman et al., 2012b). In contrast to the severe US and Taiwanese models which can be expected to only live around one week, Smn$^{2B/-}$ mice offer an opportunity to assess whether similar UPS changes are also present in less severe forms of SMA.

The presence of conserved changes across the UPS in different models of SMA would make a strong case for altered ubiquitin homeostasis being a fundamental molecular feature of reduced SMN levels and therefore a likely core regulator of disease pathogenesis. I have therefore examined if UPS alterations similar to those previously identified in SMA mice are also found in two other commonly used models of SMA: zebrafish and iPSC derived motor neurons. A zebrafish SMA model can be created by morpholino knockdown of Smn mRNA resulting in low Smn protein levels and motor axon defects (Mcwhorter et al., 2003). The use of zebrafish as a rapid genetic or drug screening tool would allow for any newly identified UPS alterations to be subsequently analysed for their influence on the SMA-like motor phenotype (Wishart et al., 2014).
iPSC derived motor neurons can be made by reprogramming patient fibroblasts (Maury et al., 2015) and offer the opportunity to investigate whether UPS changes are present in isolated motor neuron-like cells from SMA patients. This is particularly pertinent for SMA, as changes to the levels of UPS proteins in the whole organ are not required for the presence of more subtle cell-level alterations (Wishart et al., 2014). For example, Uba1 protein levels from the whole spinal cord are unchanged at a mid-symptomatic time point in Taiwanese mice even though there is a marked redistribution of Uba1 from the cytoplasm to the nucleus specifically in motor neurons at this stage of disease (Wishart et al., 2014). The use of iPSCs will, therefore, allow for the identification of clinically relevant UPS changes in isolated SMA patient motor neuron-like cells without the influence of other cell types present in the spinal cord; which may not be as susceptible as motor neurons to alterations in ubiquitin homeostasis.

3.2 Results

3.2.1 Systemic reduction of Uba1 protein levels in the Taiwanese SMA mouse model

Uba1 protein levels have been previously shown to be significantly reduced in the neuromuscular system of late-symptomatic US and Taiwanese SMA mice (Wishart et al., 2014). As a wide variety of recent evidence supports the involvement of other organ systems in SMA pathology (reviewed by (Hamilton and Gillingwater, 2013)), I wanted to establish whether Uba1 protein reduction is a global molecular feature of the disease. To do so, western blot analyses of Uba1 levels in the spinal cord, gastrocnemius muscle, heart, liver, lung and kidney were conducted at different stages of SMA phenotype progression and compared to Uba1 levels of littermate controls (N=3 for each genotype, at each age).

At the day of birth (P1), no significant alterations in Uba1 levels were noted in any of the SMA mouse organs examined (Figure 3-1C). At a pre-symptomatic time point (P3) a significant reduction in of Uba1 levels was noted in the heart (control 100% ±
9.1% vs SMA 72.6% ± 2.6%, $P<0.05$) and liver (control 100% ± 2.5% vs SMA 74.0% ± 5.6%, $P<0.05$) of SMA mice whilst other organs remained unaffected (Figure 3-1B &C). By P7 (a mid-to-late symptomatic age), a reduction of Uba1 levels was noted in all organs, apart from the spinal cord and by P11 (a very late symptomatic stage), Uba1 levels were significantly decreased in all organs (Figure 3-1C). The reduction in Uba1 levels in all organs was roughly correlated with disease progression whereby the decrease in Uba1 protein was greatest at late symptomatic time points (Figure 3-1C).

Interestingly, the organs examined were not equally affected with respect to Uba1 perturbations, with the heart displaying the greatest Uba1 protein level reduction at all time points (e.g. at P11, control 100% ± 5.6% vs SMA 36.0% ± 6.3%, $P<0.005$), followed by the liver, muscle, kidney, lung and spinal cord, which had the smallest change (e.g. at P11, control 100% ± 8.6% vs SMA 75.6% ± 7.8%, $P<0.05$ (Figure 3-1A-C).
Figure 3-1. Analysis of Uba1 protein levels in different organs of the SMA Taiwanese mouse model over time. A. Example western blot showing Uba1 levels (top panel) in the spinal cord, muscle, heart and liver of control and SMA mice at a mid-to-late symptomatic age (P7). Bottom panel shows α-tubulin loading. B. Western blots of Uba1 levels (top panels) in the heart and liver of control and pre-symptomatic age (P3). Bottom panel shows α-tubulin loading. C. Chart showing the quantification of Uba1 levels of the spinal cord, gastrocnemius muscle, heart, liver, lung and kidney of control (solid line) and SMA (dotted line) mice at P1, P3, P7 and P11. Uba1 levels are expressed as a percentage of control mouse levels and are shown as mean ± SEM. N=3 for each genotype at each time point.
3.2.2 Uba1 levels are reduced in the intermediate Smn2B/- SMA mouse model

As reduced Uba1 levels have been shown in the neuromuscular system of late-symptomatic Taiwanese and US SMA mice (Wishart et al., 2014), both severe models of SMA, I wanted to establish whether the same was true for the less severe, intermediate Smn2B/- model. Smn2B/- mice at a mid-to-late symptomatic time point (P18) are visibly smaller than wildtype controls (Smn+/+) with noticeably smaller ears and tails (Figure 3-2A), have significantly reduced body weight (Figure 3-2B) and also display a tremor and poor ability to perform the righting reflex test (data not shown). A western blot analysis of Uba1 protein levels in a variety of organs revealed that Uba1 expression was significantly reduced in tibialis anterior muscle, liver and lung of Smn2B/- mice compared to controls. Whilst no significant difference was found in the brain or kidney, there was a clear trend of reduced Uba1 levels in the spinal cord and heart of Smn2B/- mice but higher N numbers are required to achieve robust statistical significance (Figure 3-2D). In terms of Uba1 protein reduction, the liver was the most affected organ (control 100% ± 10.1% vs SMA 44.4% ± 4.3%, P<0.005). Interestingly, compared to the dark livers of control mice, the liver in the Smn2B/- mice had a white fatty appearance (data not shown) indicating the presence of a high degree of liver pathology.
Figure 3-2. Reduction in Uba1 levels in Smn2B/- mouse organs  A.
Representative photographs of wildtype (WT) and Smn2B/- at P18. B. Bar chart showing the weights of wildtype (WT) (Smn+/-) (N=3) and Smn2B/- (N=5) mice at P18. Weights displayed as mean ± SEM. ****P <0.001, unpaired, two-tailed Student’s t-test. C. Western blot of Uba1 levels (top panel) in the liver of P18 wildtype (WT) and Smn2B/- mice. Bottom panel shows α-tubulin loading. D. Graph showing the quantification of Uba1 levels in the brain, spinal cord, tibialis anterior muscle, heart, liver, lung and kidney of wildtype (WT) and Smn2B/- mice at P18. N=3 for each genotype. Data expressed as mean ± SEM. *P<0.05, ***P<0.005, ns = not significant; unpaired, two-tailed Student’s t-test.
3.2.3 A reduction in Uba1 protein levels in a zebrafish model of SMA

Pharmacological or genetic suppression of Uba1 is sufficient to recapitulate the SMA motor phenotype of Smn knockdown in zebrafish (Wishart et al., 2014). However, the question still remained as to whether SMA model zebrafish have reduced Uba1 levels? To test this, zebrafish were injected with a morpholino targeted against Smn at a mid (4ng) and high (6ng) dose at the one cell stage and harvested for protein at 48hrs post-fertilisation. Western blot analysis showed that with the 4ng morpholino injection no significant changes in Smn or Uba1 levels were present (Figure 3-3A-C). However, with the high 6ng dose where Smn levels were significantly reduced (control 100% ± 8% vs SMA 57.9% ± 0.7%, P<0.01) (Figure 3-3 A&B), Uba1 levels were also significantly lower than age matched uninjected controls (control 100% ± 15.0% vs SMA 30.9% ± 4.9%, P<0.05) (Figure 3-3 A&C) indicating that reduced Uba1 levels are a Smn dependent feature of SMA model zebrafish.
Figure 3-3. Reduction in Uba1 levels in zebrafish with Smn morpholino knockdown

**A.** Western blot showing Smn (top panel) and Uba1 (middle panel) protein levels in age matched uninjected control zebrafish and zebrafish with either 4ng or 6ng morpholino (MO) targeted against Smn. Zebrafish were injected with morpholino at the once cell stage and collected for protein analysis at 48hrs post-fertilisation. Fish were pooled in to batches of 30 fish, with three replicate batches per experimental group. Bottom panel shows COX-IV loading.

**B & C.** Bar charts showing the quantification of Smn (B) and Uba1 (C) protein levels in age matched uninjected control zebrafish and zebrafish with either 4ng or 6ng morpholino (MO) targeted against Smn. N=3 for each group. Data expressed as mean ± SEM. *P<0.05, **P<0.01, ns = not significant; unpaired, two-tailed Student’s t-test.
3.2.4 Reduced UBA1 protein levels in Type I SMA patient iPSC derived motor neurons

To test whether the reduction in Uba1 levels present in mouse and zebrafish models may be a clinically relevant molecular perturbation, I next examined whether similar alterations in UBA1 and its downstream targets were seen in Type I SMA patient iPSC derived motor neurons. Western blot analysis of unaffected control (8 independent clones) and Type I SMA (9 independent clones) iPSC derived motor neurons showed, as expected, a significant reduction in the amount of SMN protein (control 100% ± 6.7% vs SMA 39.6% ± 6.2%, P<0.001) (Figure 3-4A&B). Significantly, UBA1 protein levels also showed a robust decrease in SMA patient derived cells compared to controls (control 100% ± 8.8% vs SMA 54.8% ± 7.9%, P<0.01) (Figure 3-4A&C). Whilst not significant, a trend of increased β-catenin and UCHL1 protein levels (as present in SMA mice and patient tissue) was also observed (Figure 3-4D-E) suggesting that pathways downstream of UBA1 may also be altered in Type I SMA patient iPSC derived motor neurons.
Figure 3-4. Alterations to the UPS in Type I SMA patient iPSC derived motor neurons  

A. Western blot showing SMN and UBA1 protein levels in unaffected control and Type I SMA patient iPSC derived motor neurons. Bottom panels show β III tubulin loading.  

B-E. Bar charts showing the quantification of SMN (B), UBA1 (C), β-catenin (D) and UCHL1 (E) protein levels in unaffected control (8 independent clones) and Type I SMA patient (9 independent clones) iPSC derived motor neurons. Data expressed as mean ± SEM. **P<0.01, ***P<0.005, ns = not significant; unpaired, two-tailed Student's t-test.
3.3 Discussion

Alterations in the UPS have previously been described in the neuromuscular system of Taiwanese and ‘US’ SMA mice (Wishart et al., 2014). In this chapter I have extended these findings to show that reductions in the E1 enzyme Uba1 are found in all major organ systems of Taiwanese SMA mice and are also present in other SMA models including Smn2B/− mice, zebrafish and patient derived iPSC motor neurons.

Western blot analysis of all major organs from the Taiwanese model revealed that Uba1 protein reduction is not just restricted to the neuromuscular system and is a global molecular feature in SMA mice. The reduction in Uba1 protein levels in all organs was greater in late stage mice compared with mid- and pre-symptomatic animals, indicating that the overall degree of Uba1 reduction is correlated with disease phenotype severity. Interestingly, not all organs displayed the same degree of Uba1 protein reduction, with the heart and liver being the greatest affected at mid- and late-symptomatic stages, with detectable reduction in Uba1 even present pre-symptomatically. The spinal cord, on the other hand, was the least affected only showing a reduction in Uba1 protein levels at a late-symptomatic time points. This finding is consistent with previous reports showing that Uba1 protein levels from the whole spinal cord are unchanged at a mid-symptomatic stage even though there is a marked redistribution of Uba1 from the cytoplasm to the nucleus specifically in motor neurons at this stage of disease (Wishart et al., 2014).

For future studies it would be interesting to see if this alteration in the subcellular distribution of Uba1 is present in cells from other organs, and if so, does it precede the whole organ reduction in Uba1 levels detectable by western blot? Intriguingly, the global reduction in Uba1 levels in Taiwanese SMA mice is in contrast to the pattern of increased β-catenin expression which is only present in the neuromuscular system and stable in peripheral organs (Wishart et al., 2014). Why β-catenin levels don’t accumulate in peripheral organs even though Uba1 levels are robustly decreased is a puzzling question that remains to be investigated and further confirms
that there are fundamental differences in neuromuscular and systemic organ molecular pathology in SMA mice.

A multisystem reduction in Uba1 protein levels was also found in the milder $Smn^{2B/-}$ mice, showing that UPS alterations are also a molecular feature of intermediate forms of SMA as well as severe models. Interestingly, the profile of affected organs differed to that of the more severe Taiwanese model illustrating that the degree and pattern of Uba1 systemic reduction is model dependent. In $Smn^{2B/-}$ mice, the liver displayed the largest reduction in Uba1 protein. Out of all the organs, the livers of $Smn^{2B/-}$ appeared most grossly abnormal with a striking white discoloration. The altered gross appearance coupled with high alteration in Uba1 indicate strong liver pathology in $Smn^{2B/-}$ mice, and physiologically $Smn^{2B/-}$ livers have indeed previously been shown to display increased levels of insulin sensitivity (Bowerman et al., 2012b). Whilst no significant difference was found in the brain or kidney, in similarity with the Taiwanese model, there was a clear trend of reduced Uba1 levels in the spinal cord and heart of $Smn^{2B/-}$ mice, with further analysis necessary to increase the N numbers required for achieving robust statistical significance. Indeed, transcriptional profiling of vulnerable motor neuron populations in $Smn^{2B/-}$ mice has recently shown that there is down regulation of transcripts involved in ubiquitin metabolism, including ubiquitin itself (Murray et al., 2015), indicating Uba1 defects are likely present in the $Smn^{2B/-}$ spinal cord. The strong reduction in muscle Uba1 levels as a common feature in three independent mouse models (Taiwanese, $Smn^{2B/-}$ and previously published US mice (Wishart et al., 2014)) strongly suggests that Uba1 muscle perturbations are likely an important feature in SMA muscle atrophy.

The presence of reduced UBA1 levels in both SMA model zebrafish and Type I SMA patient iPSC derived motor neurons indicate that a reduction of UBA1 is an evolutionary conserved response to low levels of SMN protein. As zebrafish SMA models present a rapid genetic or drug screening platform it would be interesting to see if increasing levels expression of Uba1 can ameliorate Smn dependent motor axon defects. The significant reduction in UBA1 protein levels (as well as the trend for increased levels of β-catenin and UCHL1) in Type I SMA patient iPSC derived
motor neurons reveals for the first time that UBA1 dependent UPS perturbations are also present in human patient motor neuron-like cells. Importantly, this shows that the UPS alterations present in mouse and zebrafish models represent clinically relevant molecular perturbations. In the following chapters, I will therefore further examine the role of β-catenin, UCHL1 and UBA1 in SMA and explore the potential of targeting these UPS components as novel therapeutic strategies in SMA mice.
Chapter 4. Analysis of β-catenin inhibitors on the phenotype of SMA mice

4.1 Introduction

As reviewed in detail by (Valenta et al., 2012), β-catenin is a multifunctional protein involved in coordination of cell-cell adhesion and regulation of gene transcription via the Wnt (wingless-type MMTV integration site1) signalling pathway. Encoded by the CTNNBI gene in humans, β-catenin is an 88 kDa protein and was originally identified in 1990 as a component of adherens junctions (Valenta et al., 2012). Adherens junctions are protein complexes which act as a bridge to connect the actin cytoskeleton of neighbouring cells together where β-catenin is responsible for the cytoplasmic anchoring of the extracellular calcium-dependent adhesion molecule cadherin (Valenta et al., 2012). Around the same time, the components of the canonical β-catenin dependent Wnt signalling pathway were also discovered (reviewed by (Gordon and Nusse, 2006)). In the absence of an extracellular Wnt signal, β-catenin is continually targeted by its ‘destruction complex’; a protein complex comprised of: axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK-3), casein kinase 1 (CK1) and the E3-ubiquitin ligase β-TrCP (Gordon and Nusse, 2006). Phosphorylation of β-catenin by GSK3 results in a β-TrCP recognition site leading to its ubiquitination and degradation by the proteasome keeping cytoplasmic β-catenin levels low (Gordon and Nusse, 2006). Upon a Wnt ligand binding to its Frizzled transmembrane receptors and LRP5/6 co-receptor, the ‘destruction complex’ becomes destabilized meaning that β-catenin is no longer phosphorylated and degraded (Gordon and Nusse, 2006). The accumulation of cytoplasmic β-catenin leads to its translocation to the nucleus where it can associate with the TCF/Lef family of transcription factors leading to the expression of specific target genes (Gordon and Nusse, 2006).

This canonical Wnt signalling cascade is an essential pathway regulating multiple processes during embryonic development and in adult tissues. Many cancers are associated with altered Wnt signalling, and as such a great effort has been made in
identifying inhibitors of β-catenin function (Barker and Clevers, 2006). More recently, a role for Wnt/β-catenin in neurogenesis and neurodegeneration has emerged where it may play a role in the balance between neuronal survival and death in different disease states (Harvey and Marchetti, 2014). Indeed a role of dysregulated Wnt/β-catenin has been implicated in ALS (Chen et al., 2012b, Yu et al., 2013, Li et al., 2013, Pinto et al., 2013), Alzheimer’s disease (reviewed by (Boonen et al., 2009)), Parkinson’s disease (L'episcopo et al., 2011, Rawal et al., 2009) and Huntington’s disease (Godin et al., 2010).

As discussed in Chapter 1, in SMA alterations in the upstream UPS pathway (in particular loss of UBA1) means that β-catenin is no longer adequately degraded leading to its accumulation in the neuromuscular system of SMA mice and muscle biopsy samples from Type I patients (Wishart et al., 2014). Impressively, inhibition of β-catenin signalling using the plant based flavonoid compound quercetin rescued the motor neuron defects in UBA1 inhibitor treated zebrafish and reduced neuromuscular pathology in zebrafish and Drosophila SMA models (Wishart et al., 2014). β-catenin inhibition was also able to ameliorate neuromuscular pathology in severe Taiwanese SMA mice whereby quercetin robustly increased motor performance, reduced motor neuron loss, increased muscle fibre diameter and improved NMJ morphology to levels comparable to controls (Wishart et al., 2014). Given the promising effect of quercetin, in this chapter I will aim to assess if other β-catenin inhibitors also provide similar or improved enhancements in the weights, survival and motor performance of SMA mice.
4.2 Results

4.2.1 β-catenin protein levels are increased in the neuromuscular system of late-symptomatic SMA mice

In order to confirm the increased β-catenin levels in the neuromuscular system in SMA mice previously reported by Wishart et al. (2014), western blot analysis was performed on protein extracted the whole spinal cord and gastrocnemius muscle from late-stage P10 Taiwanese SMA mice and littermate controls. β-catenin protein levels in SMA tissue was found to be significantly increased in the muscle (control 100% ± 8.6% vs. SMA 135.7% ± 10.3%, N = 4, \( P < 0.05 \)) and spinal cord (control 100% ± 33.8% vs. SMA 313.8% ± 61.4%, \( N = 4, P < 0.05 \)) compared to controls (Figure 4-1A&B) which is consistent with the increase in SMA mouse tissue previously reported. No change in β-catenin levels were found in the spinal cord or gastrocnemius muscle at P7 mid-symptomatic stages (data not shown). Interestingly, as with Uba1, although β-catenin level changes in SMA were identified in hippocampal synaptosomes no changes in β-catenin levels were observed in the whole brain at either mid- or late-symptomatic stages (data not shown).

![Figure 4-1. Increased β-catenin levels in the neuromuscular system of SMA mice. A&B. Example fluorescent western blots and bar charts showing β-catenin protein levels in the spinal cord (A) and muscle (B) of P10 control and SMA mice (N=4 of each genotype, *\( P < 0.05 \), unpaired, two-tailed Student’s t-test).](image)
4.2.2 Aspirin treatment leads to adverse survival effects in control littermates at high doses and results in no improvement in the SMA mouse phenotype at low doses

Aspirin (acetylsalicylic acid) is commonly used as a prescription medication as a non-steroidal inflammatory drug due to its ability to suppress production of prostaglandins and thromboxanes due to its inhibition of cyclooxygenase 1 and 2 enzymes. Aspirin is also known to possess additional mechanisms of action, including inhibition of the canonical Wnt pathway. In particular, in vitro studies revealed that aspirin is known to inhibit Wnt signalling and target gene expression by promoting the degradation of β-catenin by inducing its phosphorylation (Dihlmann et al., 2003) and there for its ubiquitination in a PP2A dependent manner (Bos et al., 2006) as well as modulating TCF activity (Dihlmann et al., 2001). Furthermore, when delivered to a mouse model of familial adenomatous polyposis, aspirin was capable of reducing intestinal tumour growth in a mechanism likely related to its ability of reducing intracellular β-catenin levels (Mahmoud et al., 1998).

Based on the published literature of well tolerated aspirin doses delivered to mice via the intraperitoneal cavity (Stark et al., 2007, Jeong et al., 2013), a dose of 100mg/kg/day was initially chosen to be roughly comparable to high dose of aspirin in humans (Jeong et al., 2013). Daily treatment of high dose aspirin in a single Taiwanese litter (N=4 SMA mice and N=4 control littermates) was associated with a detrimental impact on survival of control littermates, even though the survival of SMA mice remained unaffected (Figure 4-2A). It was also noted that some control and SMA mice also developed slight abdominal swelling at around 8-9 days following treatment (Figure 4-2B). Mice were culled for humane reasons if this was observed. Due to these adverse effects, the dose was revised down for further experiments to be roughly comparable to an effective low dose aspirin treatment in humans (Jeong et al., 2013). At 25mg/kg/day the adverse effects noted with the high dose aspirin were prevented. However, with regards to the SMA mouse phenotype, no beneficial impact of low dose aspirin treatment was observed in all assessments made (Figure 4-2 C-G).
Whereas littermate controls put weight on steadily over time, SMA mice fail to put on weight in the same manner (Figure 4-2C). After an initial period of weight gain similar to controls, SMA mouse weights then plateau at around 2 grams before experiencing weight loss at the end stages of disease (Figure 4-2C). For all time points, weights of SMA mice with aspirin treatment not significantly different to SMA vehicle treated mice (Fig 4-2C) and no alteration in age of weight loss onset (an indicator of overt disease onset) was observed (day 9.3 for vehicle treated SMA mice, N=11 vs. day 8.8 for aspirin treated SMA mice, N=4, P>0.05) (Figure 4-2E). Survival of SMA mice also remained unaffected (median age of death of vehicle treated SMA mice was P11, N=11 vs. P12 for aspirin treated SMA mice, N=4, P>0.05).

Survival of SMA mice was also unaffected with quercetin with the beneficial impact of quercetin related to its ability to improve the neuromuscular phenotype of SMA mice. Therefore, to further assess the impact of low dose aspirin on the SMA mouse motor performance the righting reflex test was performed at days P3, P6 and P9. At P3 there was no significant difference between the righting times observed in SMA and littermate control mice (Figure 4-2F). However with disease progression, at P6 and P9 both SMA vehicle and SMA aspirin treated mice had significantly worse motor performance than control vehicle and control aspirin treated mice (for all comparisons P<0.05) (Figures 4-2F). At all three timepoints low dose aspirin administration had no significant impact on the motor performance of SMA mice (Figure 4-2F). The general appearance of SMA mice was also unaffected at all time points (Figure 4-2G).
Figure 4-2. Treatment of SMA mice with aspirin produced adverse effects at high doses and failed to ameliorate the SMA phenotype at low doses. A. Survival curves of control and SMA mice treated with 100mg/kg/day aspirin. Survival curves were compared using a log-rank (Mantel-Cox) test. B. Representative photographs of P9 control and SMA injected with vehicle or 100mg/kg/day aspirin. Arrows indicate abdominal swelling. C. Graph showing average weights of vehicle treated SMA mice and control and SMA mice injected with 25mg/kg/day aspirin. For each day postnatal age weights of SMA vehicle and aspirin treated mice were compared using a two-tailed unpaired Student’s t-test. D. Survival curves of control and SMA mice treated with 25mg/kg/day aspirin. Survival
curves were compared using a log-rank (Mantel-Cox) test. E. Bar chart showing mean day of weight loss onset of vehicle and 25mg/kg/day aspirin treated SMA mice. Groups compared using a two-tailed unpaired Student’s t-test; ns = not significant. F. Bar charts showing the righting times of vehicle and 25mg/kg/day aspirin control and SMA treated mice at P3, P6 and P9. Comparisons between control and SMA treated with vehicle or aspirin were compared using a two-tailed unpaired Student’s t-test; ns = not significant. G. Representative photographs of control and SMA injected with vehicle or 25mg/kg/day aspirin at f P3, P6 and P9.

4.2.3 Treatment with indomethacin results in no improvement in the SMA weight or motor phenotype and exacerbates survival defects.

Like aspirin, indomethacin is non-steroidal inflammatory drug with reported ability to inhibit canonical Wnt signalling. As with aspirin, indomethacin can modulate the activity of TCF resulting in the reduced transcription of β-catenin target genes (Dihlmann et al., 2001). In human colorectal cancer cells, indomethacin (unlike aspirin) is also able to reduce β-catenin protein levels (Smith et al., 2000, Hawcroft et al., 2002) and disrupt the formation of a β-catenin-TCF-DNA complex which altered TCF target gene expression (Hawcroft et al., 2002). Additionally, in a rat colorectal cancer model, indomethacin treatment was found to inhibit the translocation of β-catenin from the cytoplasm to the nucleus in tumour cells in vivo (Brown et al., 2001).

A dose of 4mg/kg/day indomethacin (Heidel et al., 2012, Shenoy et al., 2012) resulted in no difference in the weights of SMA mice at all time points (Figure 4-3A) nor did it improve the average age of weight loss onset (Figure 4-3C). There was an increase in the number of early deaths in SMA mice treated with indomethacin resulting in a significantly reduced mean survival (Figure 4-3C) (median age of death of vehicle treated SMA mice was P9, N=10 vs. P6 for indomethacin treated SMA mice, N=13, P<0.01). An examination of motor performance revealed that at all time points there was no difference in the righting times of SMA mice treated with vehicle or indomethacin (Figure 4-3D). No statistical comparison could be made between
SMA treatments at P8 as only 1 SMA mouse remained in the indomethacin group (Figure 4-3D).

**Figure 4-3.** Treatment of SMA mice with indomethacin results in no improvement in the SMA weight or motor phenotype and exacerbates survival defects. A. Graph showing average weights of vehicle treated SMA mice and control and SMA mice injected with 4mg/kg/day indomethacin. For each day postnatal age weights of SMA vehicle and aspirin treated mice were compared using a two-tailed unpaired Student's t-test. B. Survival curves of control and SMA mice treated with 4mg/kg/day indomethacin. Survival curves were compared using a log-rank (Mantel-Cox) test. **P<0.01. C. Bar chart showing mean day of weight loss onset of vehicle and 4mg/kg/day indomethacin treated SMA mice. Groups compared using a two-tailed unpaired Student's t-test; ns = not significant. D. Bar charts showing the righting times of vehicle and 4mg/kg/day indomethacin control and SMA treated mice at P3 to P8. Comparisons between control and SMA treated with vehicle or indomethacin were compared using a two-tailed unpaired Student's t-test; ns = not significant.
4.2.4 Treatment with iCRT-14 results in exacerbation of weight and survival of SMA mice but improvement of motor performance at some time points.

In addition to canonical Wnt signalling inhibition, both aspirin and indomethacin have other mechanisms of action which could be leading to the adverse effects noted both the control and SMA mice. As such, a more selective inhibitor of β-catenin signalling was next chosen to overcome this limitation. iCRT-14 is a small molecule inhibitor β-catenin responsive transcription that was initially discovered in an RNAi chemical based genetic screen (Gonsalves et al., 2011). iCRT-14 selectively inhibits β-catenin signalling by disrupting β-catenin-TCF interaction whilst having minimal impact on non-canonical Wnt signalling or other cell signalling pathways and not impacting on β-catenin’s ability to bind to its cell adhesion partners (Watanabe and Dai, 2011). In a mouse model of proliferating tumour xenografts, iCRT-14 was able to markedly reduce the initial tumour growth rate illustrating efficacy of β-catenin signalling inhibition in vivo (Gonsalves et al., 2011). Based on these efficacious doses, SMA and control littermates were administered with 20mg/kg/day to examine the effect of selective β-catenin inhibition on the SMA phenotype.

iCRT-14 treatment resulted in no significant change in the mean weights of SMA mice, although there was a trend for lower overall weight in the iCRT-14 treated group (Figure 4-4A) and was associated with a quicker onset of weight loss (day 7.3 for vehicle treated SMA mice, N=8 vs. day 5.6 for iCRT-14 treated SMA mice, N=8, $P<0.01$) (Figure 4-4C). In agreement with an exacerbated phenotype, the survival of SMA mice was slightly reduced in the iCRT-14 group as compared to controls (median age of death of vehicle treated SMA mice was P9, N=8 vs. P8 for iCRT-14 treated SMA mice, N=8, $P<0.01$). With respect to the motor performance an improvement in righting times was seen at P4 (5.7 ± 0.6 seconds for vehicle treated SMA mice vs 3.1 ± 0.5 seconds for iCRT-14 treated SMA mice, $P<0.01$) and P6 (6.0 ± 0.8 seconds for vehicle treated SMA mice vs 4.0 ± 0.91 seconds for iCRT-14 treated SMA mice, $P<0.05$) (Figure 4-4D). However these findings were inconsistent with other time points where no improvements in righting times were seen (Figure 4-4D).
Figure 4.4. Treatment with iCRT-14 results in exacerbation of weight and survival of SMA mice but improvement of motor performance at some time points. A. Graph showing average weights of vehicle treated SMA mice and control and SMA mice injected with 20mg/kg/day iCRT-14. For each day postnatal age weights of SMA vehicle and iCRT-14 treated mice were compared using a two-tailed unpaired Student’s t-test. B. Survival curves of control and SMA mice treated with 20mg/kg/day iCRT-14. Survival curves were compared using a log-rank (Mantel-Cox) test. ** P<0.01. C. Bar chart showing mean day of weight loss onset of vehicle and 20mg/kg/day iCRT-14 treated SMA mice. Groups compared using a two-tailed unpaired Student’s t-test; ** P<0.01. D. Bar charts showing the righting times of vehicle and 20mg/kg/day iCRT-14 control and SMA treated mice at P3 to P8. Comparisons between control and SMA treated with vehicle or aspirin were compared using a two-tailed unpaired Student’s t-test; ns = not significant, * P<0.05, ** P<0.01.
4.3 Discussion

Previous findings have shown that in SMA models there is an accumulation of β-catenin in the neuromuscular system, with inhibition of β-catenin signalling leading to amelioration of motor pathology (Wishart et al., 2014). In this chapter, I have confirmed the finding of increased β-catenin levels in the spinal cord and muscle of late symptomatic SMA mice and extended these findings to examine the effects of other β-catenin inhibitors on their phenotype. However, in contrast to previous beneficial results with quercetin, aspirin, indomethacin and iCRT-14 however all failed to improve the weights, survival and motor performance of SMA mice.

Aspirin was found to be associated with adverse effects and mortality of control mice at high doses. At low doses no adverse effects were seen but also there were no observed improvements in the weight, survival, motor performance and general appearance of the SMA mice. Similarly, indomethacin had no impact on the weight and motor performance of SMA mice but exacerbated the number of early deaths seen in SMA mice. Both aspirin and indomethacin are primarily non-steroidal anti-inflammatory drugs which have been associated with a high incidence of adverse effects, particularly in children where their use is contraindicated. The results in this chapter show that neonatal mice also may be particularly susceptible to their toxicity which may have outweighed any beneficial impact of their ability to inhibit β-catenin signalling in SMA mice. Indeed, the contraindication of these drugs in children would mean that, even if aspirin and indomethacin had resulted in beneficial impact in SMA, their use would only be suitable for adult Type III-IV patients.

The use of a more selective inhibitor of β-catenin signalling, iCRT-14, was chosen to overcome the off target specificity found with aspirin and indomethacin. Treatment with iCRT-14 however also led to the worsening of age of weight loss onset and mean age of survival. At some time points (P4 and P6) a significant improvement in the motor performance of SMA mice was observed although this was inconsistent with righting results from other days where no improvement was seen. Further studies could be done to refine the dose of iCRT-14 to see if a more consistent
improvement in motor performance is found without the exacerbation of weight loss and survival. Future studies should also use RT-qPCR to confirm any alterations in the SMA phenotype can be attributed to the inhibition of β-catenin responsive transcription. It may also be worth examining SMN2 mRNA levels as it has been shown that quercetin is capable of upregulating SMN2 transcription in patient fibroblasts (Uzunalli et al., 2015) which may have contributed to the beneficial impact of quercetin previously reported in SMA mice.

How may dysregulated β-catenin signalling play a role in SMA pathogenesis? As reviewed by (Purro et al., 2014), Wnt signalling plays an important role in normal synaptic formation, function and maintenance. As β-catenin accumulation is present in synaptosomes of presymptomatic SMA mice (Wishart et al., 2014), it likely stands that the dysregulation of β-catenin signalling contributes to the early synaptic pathology of SMA, particularly in the neuromuscular system where β-catenin accumulation is greatest. β-catenin activity has also been shown to be important for NMJ formation, where overexpression of stable β-catenin in the muscle (but interestingly not in motor neurons) led to extensive outgrowth and branching of spinal axons and ectopic muscle innervation (Wu et al., 2012). Conversely, deletion of muscle β-catenin also caused NMJ defects such as mislocalisation of AchR clusters and nerve branches along with altered electrophysiological function (Li et al., 2008). Together these studies indicate an intricate balance of β-catenin levels in muscle is required for the normal development and functions of NMJs, which are a known early target in SMA.

Further lessons could be learnt from other neurodegenerative conditions associated with β-catenin signalling disruption. In the SOD1(G93A) mouse model of ALS, an increase in Wnt signalling components and target genes was found to coincide with the onset of symptoms (Yu et al., 2013, Li et al., 2013). In the same model there is also a translocation of β-catenin from the cytoplasm to the nucleus of motor neurons associated with increased transcription of downstream target genes (Chen et al., 2012b). Expression of β-catenin was also found to be increased in spinal astrocytes which were associated with glial proliferation (Chen et al., 2012b). Translocation and
aggregation of β-catenin was also noted in ALS-like NSC34 cells expressing hSOD1(G93A) (Pinto et al., 2013). Interestingly, riluzole is able to regulate β-catenin signalling in a cell model of melanoma (Biechele et al., 2010).

In Alzheimer’s disease the situation is less clear with reports of both up- and down-regulation of β-catenin signalling being associated with the disorder (Boonen et al., 2009). Presinilins are known to regulate the stability of β-catenin with disease associated mutations in the presenilin 1 gene interrupting the interaction of β-catenin and GSK-3 thereby reducing the constitutive turnover of β-catenin (Kang et al., 1999). Genetic variations in low-density lipoprotein receptor-related protein 6 (LRP6) (De Ferrari et al., 2007) and apolipoprotein E (Caruso et al., 2006) are however are generally associated with Wnt signalling inhibition. Parkin, an E3 ligase linked to Parkinson’s disease, is able to regulate β-catenin with upregulated β-catenin levels found in the midbrains of parkin knockout mice (Rawal et al., 2009). In primary neuron culture stabilization β-catenin and activation of Wnt signalling caused increased incidence of cell death suggesting parkin protects dopaminergic neurons from excessive canonical Wnt signalling (Rawal et al., 2009). However, conversely in a MTPT mouse model of Parkinson’s disease, activation of β-catenin with a GSK-3 antagonist was found to promote the restoration of dopaminergic midbrain neurons resulted in an associated improvement in motor performance (L’episcopo et al., 2014). In Huntington’s disease, mutant huntingtin protein abnormally associates with the β-catenin degradation complex leading to the toxic accumulation of phosphorylated β-catenin (Godin et al., 2010). Promoting the degradation β-catenin in a Huntington’s primary neuronal and Drosophila models was found to be neuroprotective, with indomethacin treatment also increasing the lifespan of Drosophila mutants (Godin et al., 2010). Taken together these studies show that attempts to restore β-catenin homeostasis in SMA could also be applicable to a wide variety of neurodegenerative diseases.
Chapter 5. Analysis of Uchl1 inhibition in SMA mice

5.1 Introduction

Ubiquitin carboxyl-terminal esterase L1 (UCHL1) is a highly conserved deubiquitinating enzyme (DUB) that acts as a thiol protease to release monoubiquitin from polyubiquitin chains or ubiquinated proteins by hydrolysing peptide bonds at the C-terminal glycine of ubiquitin (Meray and Lansbury, 2007) (Figure 1-3). However, the complex function of UCHL1 means it can also act as a monoubiquitin stabiliser (Osaka et al., 2003) and under certain circumstances possess opposing ligase activity (Liu et al., 2002). UCHL1 is strongly expressed in neurons (Day and Thompson, 2010) and is one of the most abundant proteins in the brain (Wilkinson et al., 1989). Importantly, UCHL1 has been implicated in the pathology of several neurodegenerative disorders including Alzheimer’s disease (Xue and Jia, 2006, Gong et al., 2006), Parkinson’s disease (Leroy et al., 1998, Wintermeyer et al., 2000, Maraganore et al., 2004, Facheris et al., 2005, Snapinn et al., 2011) and an early onset progressive neurodegenerative syndrome (Bilguvar et al., 2013).

As discussed previously, Uch1 levels have been shown to be robustly increased in hippocampal synaptic preparations from severe SMA mice (Wishart et al., 2014) and in SMA patient fibroblasts where knock-down or inhibition of UCHL1 led to increased SMN levels (Hsu et al., 2010). Together these studies indicate that increased UCHL1 levels may be contributing to SMA disease pathogenesis and that inhibiting UCHL1 in vivo may offer an attractive therapeutic strategy. Therefore, in this chapter I will aim to further investigate the role of UCHL1 in SMA pathology and establish whether its pharmacological inhibition can improve, survival, motor symptoms and neuromuscular pathology of SMA mice.
5.2 Results

5.2.1 UCHL1 levels are increased in Type I SMA patient fibroblasts

In order to confirm the increased UCHL1 levels in patient cells previously reported by Hsu et al. (2010), western blot analysis was performed on protein extracted from fibroblasts from 3 Type I SMA patients and age-matched, unaffected controls (Figure 5-1). UCHL1 protein levels in SMA patient cells were found to be significantly increased compared to controls (control 100% ± 48.8% vs. SMA 6000% ± 671.9&; N=3, P<0.01) (Figure 5-1A&B) which is consistent with the large increase in SMA patient fibroblasts as previously reported (Hsu et al., 2010).

![Figure 5-1. Increased UCHL1 levels in Type I SMA patient fibroblasts. A.](image)

Fluorescent western blot of UCHL1 protein levels in cultured primary fibroblasts from three individual Type I SMA patients and age matched controls. Total protein levels as indicated by Ponceau S staining were used as a loading control. Lane order: control ML-32, SMA ML-83, control ML-44, SMA ML17, control ML-24 and SMA ML-16. B. Quantification of control and SMA fibroblast UCHL1 protein levels (N=3 of each genotype, **P <0.01, unpaired, two-tailed Student's t-test).
5.2.2 Uchl1 levels are increased in SMA mouse spinal cord and muscle

Alongside reports from SMA patient fibroblasts, Uchl1 levels have been shown to be robustly increased hippocampal synaptic preparations from severe SMA mice (Wishart et al., 2014). I therefore wanted to confirm if Uchl1 protein levels are also increased in the neuromuscular system of the Taiwanese mouse model. Western blot analysis of P10 SMA and littermate control tissue revealed that the Uchl1 levels were significantly elevated in the spinal cord (control 100% ± 9.1%, N=4 vs. SMA 269.0% ± 18.3%, N=4, P<0.005) (Figure 52A&B) and gastrocnemius muscle (control 100% ± 12.1%, N=3 vs. SMA 304.3% ± 41.2%, N=4, P<0.01) (Figure 5-2D and E). An increase in Uchl1 could also be detected by immunohistochemistry in the ventral horn of the spinal cord (Figure 5-2C) and gastrocnemius muscle where the elevated Uchl1 muscle staining correlated with the location of endplate bands (Figure 5-2F). Interestingly, no overt change in Uchl1 protein level was detected in the whole brain (Figure 5-2G&H) although a change in Uchl1 distribution was noted in certain SMA brain areas including the Purkinje layer of the cerebellum where Uchl1 staining was more diffuse (Figure 5-2I). A time course analysis of Uchl1 protein levels in the gastrocnemius muscle showed that although no change was detected at pre-symptomatic age (P1), the increase in Uchl1 levels with respect to control correlated with disease severity increasing from mid-symptomatic (P4/P7) to late-symptomatic stages (P10) (Figure 5-2J).
Figure 5-2. Increased Uchl1 levels in the Taiwanese SMA mouse neuromuscular system. A, D & G. Western blot analysis of Uchl1 levels in the spinal cord (A), gastrocnemius muscle (D) and whole brain (G) of P10 SMA mice and control littermates. Total protein loading controls as indicated by Ponceau S. B, E & H. Quantification of Uchl1 western blot analysis (N=3 of each genotype, ***P < 0.005, **P <0.01, ns = not significant unpaired, two-tailed Student’s t-test). C, F & I. Uchl1 immunohistochemistry in the spinal cord ventral horn (C), gastrocnemius muscle (acetylcholine receptor endplates shown in green) (F) and cerebellum (I) of P10 control and SMA mice. J. Temporal analysis of Uchl1 protein levels in the gastrocnemius muscle at P1, P4, P7 and P10 (N=3 of each genotype, *P < 0.05, **P <0.01, ns = not significant unpaired, two-tailed Student’s t-test).
5.2.3 Pharmacological inhibition of UchL1 in vivo reduced ubiquitin levels in the SMA mouse spinal cord but did not affect SMN levels

In order to establish whether inhibiting UchL1 is an effective therapeutic strategy for SMA, SMA mice and control littermates were dosed twice daily with 0.5 mg/Kg LDN-57444 (or vehicle only control) via IP injection, starting at the day of birth. LDN-57444 is a potent pharmacological inhibitor of UchL1 that has previously been used in in vivo rodent studies where treatment has been shown to significantly reduce ubiquitin levels in the CNS (Cartier et al., 2012), with measurement of monoubiquitin levels established as a good readout marker for UchL1 in vivo activity (Cartier et al., 2009). As such, monoubiquitin levels were measured by western blot analysis in spinal cord tissue taken from LDN-57444 treated SMA mice at P10. LDN-57444 treatment significantly reduced levels of monoubiquitin compared to control (vehicle 100% ± 4.22%, N=3 vs. LDN-57444 84.23% ± 5.89%, N=3, P<0.05) (Figure 5-3A&B) indicating that the dosing regime was effective at inhibiting UchL1 hydrolase activity in SMA mice in vivo. However, in contrast to previous SMA fibroblast in vitro data (Hsu et al., 2010), pharmacological inhibition of UchL1 did not increase SMN levels in vivo in SMA mice (vehicle 100% ± 22.83% vs. LDN-57444 99.29% ± 28.53%, N=3) (Figure 5-3C&D).
Chapter 5. Analysis of Uchl1 inhibition in SMA mice

5.2.4 Pharmacological inhibition of Uchl1 did not improve the weight or survival of SMA mice, and precipitated the onset of weight loss

To determine whether pharmacological inhibition of Uchl1 would ameliorate the phenotype of SMA mice an analysis of weight and survival was undertaken. Treatment with LDN-57444 did not modify the average daily weight of SMA mice (Figure 5-4A). However, the age at which weight loss was first observed (an indicator of overt disease onset) was significantly reduced in the LDN-57444 treated SMA mice, suggesting that Uchl1 inhibition actually had a detrimental impact on disease pathology ($P<0.05$) (Figure 5-4C). The survival curve of LDN-57444 treated mice was shifted to the left of the vehicle treated curve (Figure 5-4B), indicating that LDN-57444 was precipitating the onset of disease, although the median survival did not reach statistical significance (median age of death of vehicle treated SMA mice was P11, N=11 vs. P10 for LND-57444 treated SMA mice, N=10) (Figure 5-4B).
LDN-57444 treated control littermates showed no detrimental impact on weight or survival (Figure 5-4A&B).

**Figure 5-4. Pharmacological inhibition of Uchl1 failed to improve weight or survival of SMA mice, and precipitated the onset of weight loss.** A. Graph showing average weights of vehicle treated and LDN-57444 treated SMA and control mice. For each day postnatal age weights of SMA vehicle and LDN-57444 treated mice were compared using a two-tailed unpaired Student’s t-test. B. Survival curves showing percentage survival of vehicle treated and LDN-57444 treated SMA and control mice. Survival curves were compared using a log-rank (Mantel-Cox) test. Median age of death of vehicle treated SMA mice P11, N=11 vs. P10 for LND-57444 treated SMA mice, N=10. C Bar chart comparing onset of weight loss in vehicle treated SMA mice (N=11) with LDN-57444 treated SMA mice (N=10). Data shown as mean ± SEM, * P < 0.05; two-tailed unpaired Student's t-test.

**5.2.5 Pharmacological inhibition of Uchl1 did not improve motor performance of SMA mice**

To further assess the impact of Uchl1 inhibition on the SMA phenotype the righting reflex test to assess motor performance was performed at days P3, P6 and P9. At all three time points LDN-57444 administration had no significant impact on the motor performance of SMA mice (Figure 5-5A-C). Thus, treatment with LDN-57444 had
no measurable effect on motor performance in SMA mice. The general appearance of SMA mice was also unaffected at all time points (Figure 5-5D-F).

Figure 5-5. Pharmacological inhibition of Uchl1 had no effect on motor performance or appearance of SMA mice. A-C. Bar charts showing the righting time taken for control vehicle treated mice, control LDN-57444 treated mice, SMA vehicle treated mice and SMA LDN-57444 treated mice at (A) P3, (B) P6 and (C) P9. Data are shown as mean ± SEM. Control vehicle treated mice P3 N=19, P6 N=16, P9 N=16. Control LDN-57444 treated mice P3 N=15, P6 N=12, P9 N=12. SMA vehicle treated mice P3 N=17, P6 N=15, P9 N=11. SMA LDN-57444 treated mice P3 N=10, P6 N=9, P9 N=6. Comparisons between each group at each time point were made using ANOVA with Turkey’s post hoc comparisons test (ns = not significant). D-F. Representative photographs of control vehicle, SMA vehicle and SMA LDN-57444 treated mice are shown at (D) P3, (E) P6 and (F) P9.

5.2.6 Pharmacological inhibition of Uchl1 did not improve the neuromuscular pathology of SMA mice.

In order to assess whether Uchl1 inhibition was having a sub-clinical impact on the neuromuscular pathology of SMA mice analysis of lumbar spinal cord motor neurons, muscle fibre diameters and NMJ axonal inputs were analysed. Consistent with previous reports (Riessland et al., 2010) in the Taiwanese model at a late symptomatic time point (P8) there was a small but significant reduction in low motor
neuron cell body numbers (Figure 5-6A&B). Treatment with LDN-57444 did not ameliorate motor neuron loss in SMA mice (SMA+vehicle 15.5 ± 0.2, N=4 vs. SMA+LDN-57444 15.1 ± 0.4, N=4) (Figure 5-6A&B). P8 late-symptomatic SMA mice have a reduction in average muscle fibre diameters compared to control littermates (Figure 5-6C-E). With LDN=54=7444 treatment these was no improvement in TVA and LAL muscle fibre width in TVA muscles (SMA+vehicle 21.1 ± 0.5um, N=4 vs. SMA+LDN-57444 21.1 ± 0.3um, N=4) and LAL muscles (SMA+vehicle 21.1 ± 0.7um, N=4 vs. SMA+LDN-57444 20.8 ± 0.1um, N=4) (Figure 5-6C-E). Similarly, LDN-57444 treatment did not ameliorate the modest loss of axonal inputs to the NMJ seen in SMA mice compared to controls (SMA+vehicle 1.4 ± 0.04um, N=4 vs. SMA+LDN-57444 1.4 ± 0.02um, N=4) (Figure 5-6F-G).
Figure 5-6. Pharmacological inhibition of Uchl1 did not improve the neuromuscular pathology of SMA mice. A. Representative phase contrast micrographs of Nissl stained motor neuron cell bodies in the ventral grey horn of spinal cords from SMA and littermate controls mice at P8 treated with LDN-57444 or vehicle control. B. Bar chart comparing motor neuron soma in SMA and littermate controls mice at P8 treated with LDN-57444 or vehicle control (N=4 mice per treatment group). C. Representative phase contrast micrographs of individual muscle fibres from the TVA muscle from SMA and littermate controls mice at P8 treated with LDN-57444 or vehicle control. D-E. Bar charts comparing muscle fibre diameters of TVA (D) and LAL (E) muscles from SMA and littermate controls mice at P8 treated with LDN-57444 or vehicle controls. F. Representative confocal images of NMJ immunohistochemistry from the caudal band of the LAL muscle from SMA and littermate controls mice at P8 treated with LDN-57444 or vehicle control. Green = axons and pre-synaptic nerve terminals. Red = postsynaptic acetylcholine receptors. G. Bar chart comparing the number of axonal NMJ inputs in the caudal band of the LAL from SMA and littermate controls mice at P8 treated with LDN-57444 or vehicle control. Comparisons between each group were made using ANOVA with Turkey’s post hoc comparisons test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns = not significant.
5.2.7 Uba1 inhibition increased Uchl1 levels \textit{in vitro}

The observation that pharmacological inhibition of Uch11 exacerbated, rather than improved, the health of SMA mice, alongside the observation that inhibition of Uch11 led to a decrease in monoubiquitin levels \textit{in vivo} (thereby phenocopying ubiquitination defects previously reported in SMA mice (Wishart et al., 2014)) led to the possibility that increased levels of Uch11 in SMA actually represent an attempted compensatory response, rather than driving neuromuscular pathology. I therefore wanted to establish whether increased levels of Uch11 were caused by upstream changes in other aspects of the ubiquitin pathway known to be altered in SMA.

Given that we previously demonstrated that low levels of SMN lead to reduced levels of Uba1 in SMA mice, and that genetic or pharmacological suppression of Uba1 is sufficient to recapitulate SMA-like neuromuscular pathology in zebrafish (Wishart et al., 2014), I examined whether increased levels of UCHL1 are a result of lowered Uba1 levels.

Primary hippocampal neurons were therefore treated with UBEI-41, a pharmacological Uba1 inhibitor (Satheshkumar et al., 2009, Rinetti and Schweizer, 2010), for 2 hours and the effect on Uch11 levels examined. Cells treated with UBEI-41 had significantly reduced monoubiquitin levels compared to untreated controls (Figure 5-7A&B). Similarly, Uch11 levels were robustly increased in cells treated with UBEI-41 (Figure 5-7A&C). Thus, suppression of Uba1 was sufficient to robustly increase Uch11 levels, suggesting that the increased levels of Uch11 observed in SMA are a downstream consequence of Uba1 suppression, and likely represent an attempted compensatory response.
5. Discussion

Previous findings from animal models and primary patient fibroblasts suggested that inhibition of UCHL1 might offer an attractive therapeutic approach for the treatment of SMA (Hsu et al., 2010, Wishart et al., 2014). In this chapter I have confirmed the finding that UCHL1 levels are robustly increased in Type I SMA patient fibroblasts and extend these observations by showing that Uchl1 levels are significantly increased in the neuromuscular system in SMA mice. However, in contrast to the initial suggestions that inhibition of UCHL1 might represent a therapeutic target for SMA, pharmacological inhibition of Uchl1 in SMA mice failed to improve survival or motor symptoms or neuromuscular pathology, and instead precipitated the onset of weight loss. This worsening of overt disease status could potentially be explained by the observation that pharmacological inhibition of Uch1 in vivo significantly decreased spinal cord monoubiquitin levels, thereby exacerbating ubiquitination defects previously reported in SMA mice. This finding, together with the known DUB role of Uch1, suggests that the increased Uch1 levels present in SMA actually represent an attempted compensatory response to help increase and restore ubiquitin...
homeostasis. I also show that pharmacological inhibition of Uba1 primary neuronal cell culture, thereby phenocopying the situation observed in SMA, was sufficient to robustly increase Uchl1 expression in vitro. This indicates that the increased levels of Uchl1 observed in SMA are, at least in part, a downstream consequence of Uba1 suppression and are a response to upstream defects in ubiquitin homeostasis previously shown to be caused by low levels of SMN (Wishart et al., 2014).

Maintenance of ubiquitin homeostasis is crucial to nervous system development and function (Mayer, 2003) with DUBs, such as UCHL1, playing crucial roles in the conservation of ubiquitin levels by preventing inappropriate ubiquitin degradation by the proteasome (Chen et al., 2011). The importance of UCHL1 activity is underscored by the association of several UCHL1 loss-of-function mutations in neurodegenerative diseases and the neurodegenerative phenotypes of experimental models of UCHL1 inhibition. The G7A missense mutation in the UCHL1 gene reduces ubiquitin binding affinity causing a near complete loss of UCHL1 hydrolytic activity and is linked to a severe early-onset neurodegenerative syndrome (Bilguvar et al., 2013). Similarly, the I93M UCHL1 mutation, which confers a 50% reduction in catalytic activity, has been reported in a family with autosomal dominant Parkinson’s disease (Leroy et al., 1998) with expression of the I93M mutant in transgenic mice leading to the degeneration of dopaminergic neurons (Setsuie et al., 2007). Conversely, the S18Y polymorphism variant of UCHL1, which has comparable hydrolase but reduced ligase activity, has been associated with a decreased Parkinson’s disease risk (Liu et al., 2002, Elbaz et al., 2003). Down-regulation of UCHL1 has been reported in Alzheimer’s disease brains where the number of neurofibrillary tangles is inversely proportional to soluble UCHL1 levels (Choi et al., 2004). In the APP/Ps1 mouse model of Alzheimer’s Uchl1 has been shown to be important in synaptic function and contextual memory (Gong et al., 2006). In vitro application of LDN-57444 to hippocampal slices reduced long-term potentiation in hippocampal whilst transduction of Uchl1 protein corrected defects in synaptic transmission and improved fear learning in vivo (Gong et al., 2006).

However, other studies have found potential beneficial effects of pharmacological inhibition of Uchl1 by LDN-57444 for Alzheimer’s treatment by enhancing
clearance and reducing alpha-synuclein levels (Cartier et al., 2012). Interestingly, in non-transgenic animals and neuronal cells with endogenous Uchl1 levels LDN-57444 treatment produced the opposite effect on alpha-synuclein distribution, highlighting the different effects Uchl1 inhibition under normal and pathological conditions (Cartier et al., 2012).

Uchl1 knock-out mice present with progressive paralysis and premature death associated with progressive degeneration of pre-synaptic terminals and marked impairment of synaptic transmission at the neuromuscular junction (NMJ) (Chen et al., 2010a). Similarly, ubiquitin deficiency caused by loss of the DUB Usp14 in ataxia mice has been shown to cause selective developmental defects at the motor endplate and disruption of synaptic transmission, with transgenic restoration of ubiquitin levels preventing pathology (Chen et al., 2011). These striking similarities of NMJ vulnerability due to the loss of DUBs could be due the high demand of the UPS in endplate development coupled with the long distances of ubiquitin transport making motor neurons particularly vulnerable to local fluctuations in ubiquitin levels (Chen et al., 2011). As discussed previously, NMJ denervation caused by loss of presynaptic inputs is also observed in early symptomatic SMA mice (Murray et al., 2008). A spontaneous mutation in the UchL1 gene, which causes loss of detectable Uchl1 protein expression, is present in gracile axonal dystrophy (gad) mice (Saigoh et al., 1999). Gad mice display progressive accumulation ubiquitinated protein conjugates alongside axonal degeneration of the gracile tract along with motor paresis (Kwon and Wada, 2006).

Recently, it has also been shown that eGFP expression driven by the UchL1 promoter genetically labels a subpopulation of degeneration resistant spinal motor neurons in a mouse model of ALS (Yasvoina et al., 2013), indicating that increased Uchl1 levels may in fact be neuroprotective. Furthermore, overexpression of UCL1 induced by hippocampal injection of AAV1-UCHL1 in a APP23/PS45 Alzheimer’s mouse model was able to decrease brain plaque formation and improve learning and memory deficit (Zhang et al., 2014). Conversely, reducing Uchl1 levels by crossing APP23/PS45 mice with gad mice increased the rate of plaque formation (Zhang et
al., 2014). In light of these recent studies, my findings indicate that the increased UCHL1 levels in SMA may therefore represent an attempted compensatory protective response to defects occurring elsewhere in the UPS. Such a scenario would help explain the detrimental impact of inhibiting Uch1 hydrolase activity presented in this chapter. Thus, further studies examining the influence of increased levels of UCHL1 in SMA may now be warranted. However, targeting aspects of UPS dysfunction that are directly contributing to disease pathogenesis (such as suppression of Uba1) may prove to be more beneficial in the long run for developing novel SMA therapeutics.
Chapter 6. Developing UBA1 gene therapy for SMA

6.1 Introduction

UBA1 is an E1 activating enzyme catalysing the first step in ubiquitination, in which cellular proteins are subsequently marked for degradation. As reviewed in detail by Schulman and Harper (2009), in this process UBA1 first binds ubiquitin, and in an ATP dependent manner, catalyses the C-terminal adenylation of ubiquitin. The reactive cysteine residue in the catalytic domain of UBA1 then attacks this bond to form a high-energy thioester linkage between UBA1 and the now activated ubiquitin molecule (Schulman and Harper, 2009). UBA1 subsequently catalyses the adenylation of a second ubiquitin and becomes ‘doubly loaded’ with two ubiquitin molecules at different active sites: one covalently linked by thioester at its cysteine residue and another non-covalently associated to the adenylation active site (Schulman and Harper, 2009). Such ‘double loading’ of ubiquitin to UBA1 is thought to make the subsequent interaction with its E2 conjugating enzyme more energetically and conformationally favourable (Schulman and Harper, 2009). The activated ubiquitin is then transferred to the E2’s catalytic cysteine residue via thioesterification (Schulman and Harper, 2009). The ubiquitin charged E2 enzyme recognises E3 ligase enzymes which are attached to their substrates allowing ubiquitin chains to be built up which signals the substrate’s degradation by the proteasome (Schulman and Harper, 2009).

UBA1 is one of only two E1 enzymes present in humans (the other being UBA6) and is the ubiquitin activating enzyme that accounts for the vast majority of cellular ubiquitination events (reviewed by (Groettrup et al., 2008)). The human UBA1 gene is 24,329 bases in size, and in all mammals is encoded on the X chromosome. Alternatively spliced transcript variants exist; the two main ones being UBA1a (1058 amino acids) and UBA1b (1018 amino acids) (Stephen et al., 1997). An alternate start site means that UBA1b lacks the four serine residues required for UBA1 phosphorylation and a nuclear localisation signal that UBA1a possess, meaning that the two protein isoforms have different preferential cellular localisations: UBA1a
predominantly nuclear and UBA1b predominantly cytoplasmic (Stephen et al., 1997). As well as its role in protein degradation, UBA1 is important for other ubiquitin dependent cellular functions, including cell cycle progression (Grenfell et al., 1994, Sudha et al., 1995, Jin et al., 2007, Lee et al., 2008, Sugaya et al., 2014), autophagy (Chang et al., 2013), apoptosis (Pfleger et al., 2007, Lee et al., 2008) and repair of DNA damage (Nouspikel and Hanawalt, 2006, Moudry et al., 2012).

In 2008, the genetic cause of an X-linked form of SMA was identified as mutations in the UBA1 gene firmly implicating UBA1 in infantile motor neuron diseases (Ramser et al., 2008). As discussed in Chapter 1, UBA1 is also at the apex of an exciting new mechanistic link between SMN and the dysregulation of ubiquitin homeostasis in autosomal SMN-dependent SMA (Wishart et al., 2014). Initially discovered in a proteomic analysis of pre-symptomatic SMA mouse synaptosomes, reduced levels of UBA1 were confirmed to be a key molecular feature of the neuromuscular system in late-symptomatic US and Taiwanese models (Wishart et al., 2014). SMN and UBA1 proteins physically interact and importantly, considering the role of SMN in mRNA processing, the splicing of Uba1 mRNA is altered in the late-symptomatic Taiwanese SMA spinal cord (Wishart et al., 2014). Suppression of Uba1 in zebrafish using a morpholino or pharmacological agents led to SMA-like motor axon branching abnormalities with truncated or missing motor neurons present at high drug doses, illustrating that Uba1 suppression is sufficient to recapitulate a SMA motor-neuron phenotype in vivo (Wishart et al., 2014). Suppression of Uba1 in cell-culture or zebrafish was found to be sufficient to greatly increase downstream β-catenin levels, with inhibition of β-catenin capable of ameliorating the neuromuscular deficits in SMA mice (Wishart et al., 2014), showing that targeting this pathway is a promising therapeutic strategy for future studies.

In Chapter 3, I have extended these findings to show that to show that reductions in Uba1 are found in all major organ systems of Taiwanese SMA mice and are also present in other SMA models including Smn2B/− mice, zebrafish and human patient-derived iPSC motor neurons. These findings show that Uba1 protein reduction is not just restricted to the neuromuscular system but also represents a systemic molecular feature of both severe and intermediate SMA mice. The presence of reduced Uba1
levels in both SMA model zebrafish and Type I SMA patient iPSC derived motor neurons indicate that a reduction of Uba1 is an evolutionary conserved response to low levels of SMN protein. The significant reduction of UBA1 protein levels in Type I SMA patient iPSC derived motor neurons importantly shows that the UBA1 alterations present in mouse and zebrafish models represents a clinically relevant molecular perturbation. In Chapter 5, I also showed that suppression of Uba1 is sufficient to robustly increase Uchl1 and decrease mono-ubiquitin levels suggesting other UPS alterations present in SMA are a downstream consequence of Uba1 suppression.

Taken together these data show strong experimental evidence that reduced Uba1 levels are a conserved molecular feature of SMA and that reduced Uba1 activity directly contributes to SMA pathogenesis. In this chapter I have therefore aimed to assess if increasing UBA1 levels using gene therapy is an effective and safe therapeutic strategy in SMA mice.

6.2 Results

6.2.1 Intraperitoneal delivery of AAV9-UBA1 leads to an increase in UBA1 protein levels in peripheral organs of SMA mice

Initially, I wanted to establish whether UBA1 gene therapy was capable of increasing UBA1 levels in SMA mice in vivo. AAV9 vectors containing a full-length human UBA1 ORF cDNA construct were made by Eva Karyka in the laboratory of Professor Mimoun Azzouz at the University of Sheffield. Due to the large size of the UBA1 gene and limited coding capacity of self-complementary AAV (scAAV), constraints of vector packaging meant that conventional AAV9 was required for manufacture. Intraperitoneal injection of AAV9-UBA1(2.4xE+11 vg, 30ul injection volume) to a TTG litter at the day of birth (P1) led to the robust increase of UBA1 protein levels in the muscle, heart, liver and lung tissue of SMA mice culled at P8 compared to uninjected age matched SMA organs (Figure 6-1B). Western blot analysis showed that the increase in UBA1 levels was greatest in the heart.
(uninjected SMA 100% ± 12.8% vs. SMA AAV9-UBA1 1888.5% ± 712.9%, N=3 per group, \( P<0.05 \)) (Figure 6-1A & B), followed by liver, skeletal muscle and lung. A modest increase (~10-20%) in UBA1 levels was also detected in the brain, spinal cord and kidneys, but these changes did not reach statistical significance (Figure 1B). These data importantly established that AAV9-UBA1 gene therapy is capable of increasing UBA1 levels in SMA mice and that intraperitoneal delivery leads to the preferential targeting of peripheral organs and skeletal muscle.

Figure 6-1. Increase in UBA1 protein levels in peripheral organs and skeletal muscle of P8 SMA mice following an intraperitoneal injection of AAV9-UBA1 at P1. A. Western blot showing UBA1 protein levels (top panel) in the heart of P8 uninjected SMA mice and P8 SMA mice which had received an intraperitoneal injection of AAV9-UBA1 at P1. Bottom panel shows α-tubulin loading. B. Bar charts showing the quantification of UBA1 protein levels in the brain, spinal cord, gastrocnemius muscle, heart, liver, lung and kidney of P8 uninjected SMA mice and P8 SMA mice which had received an intraperitoneal injection of AAV9-UBA1 at P1. UBA1 levels are expressed as a percentage of uninjected SMA levels and are shown as mean ± SE. N=3 for each treatment group. \( *P<0.05 \), ns = not significant; unpaired, two-tailed Student’s t-test.

6.2.2 Intraperitoneal injection of AAV-UBA1 is safe in control mice in the long-term

As UBA1 gene therapy has not been characterised in mice before, and considering the importance of UBA1 function on the UPS and other cellular processes, I next wanted to see if UBA1 overexpression was safe over a long-term period in control mice.
mice. The health, weight, survival and motor performance of littermate controls following UBA1 gene therapy was therefore closely monitored. Similarly to SMA mice, AAV9-UBA1 injection at P1 lead to a robust increase in UBA1 protein levels in the heart at P8 (Figure 6-2A&B), an increase which was maintained at 1 month (Figure 6-2A&B) and 2 months of age (Figure 6-2A&B). Over a 2 month period, AAV9-UBA1 gene therapy had no adverse impact on the survival (Figure 6-2C), weight (Figure 6-2D) or motor performance (Figure 6-2E) of control mice. The absence of overt adverse effects following intraperitoneal AAV9-UBA1 injection at P1 illustrates that increasing UBA1 levels in vivo is safe and well tolerated in mice over a long-term period.

Figure 6-2. No overt adverse effects noted in control mice treated mice which received an intraperitoneal injection of AAV9-UBA1 at P1. A. Western blot showing UBA1 protein levels (top panels) in the heart of P8, P30 and P60 uninjected control mice and control mice which had received an intraperitoneal injection of AAV9-UBA1 at P1. Bottoms panel shows α-tubulin loading. B. Bar charts showing the quantification of UBA1 protein levels (top panels) in the heart of P8, P30 and P60 uninjected control mice and
control mice which had received an intraperitoneal injection of AAV9-UBA1 at P1. UBA1 levels are expressed as a percentage of uninjected control levels and are shown as mean ± SEM. N=3 for each genotype. C-E. Graphs showing the survival curve (C), weights (D) and righting times (E) of uninjected control (N=10 at P1-P8, N=7 at P30, N=3 at P60) and control mice treated control mice which had received an intraperitoneal injection of AAV9-UBA1 at P1 (N=13 at P1-P8, N=6 at P30, N=3 at P60. Reduction in N numbers over time due to mice being culled at each time point for tissue analysis.

6.2.3 Intraperitoneal injection of AAV9-UBA1 improves the weight and motor performance but not survival of SMA mice

I next wanted to establish whether increasing UBA1 levels using intraperitoneal delivery of gene therapy could improve the phenotype of SMA mice. Injection of a TTG litter (N=5 SMA and N=7 control littermates) at P1 with AAV9-UBA1 revealed that there was a clear trend in improvement in the weights of the SMA compared to uninjected controls at mid- to late-symptomatic stages (Figure 6-3A). For example, at P8 uninjected SMA mice weighed 1.7g ± 0.1g whereas UBA1 gene therapy mice weighed 2.2g ± 0.2g, which was heavier than the peak weight of uninjected SMA mice before weight loss onset (Figure 6-3A). The motor performance of SMA mice also showed improvement, with a trend of quicker righting times a late-symptomatic ages (Figure 6-3B). At P7, this improvement in righting performance reached statistical significance (7.6s ± 1.3s SMA uninjected vs. 3.1s ± 0.6s SMA + AAV9-UBA1, P<0.05) with UBA1 gene therapy SMA mice returning to the performance level of littermate controls (P>0.05). Analysis of survival, showed no significant improvement (Figure 6-3C), although median survival for uninjected SMA mice was P9 (N=5), compared to P10 for UBA1 gene therapy SMA mice (N=5). Injection with AAV9-UBA1 had no detrimental impact on the overall appearance of either control or SMA mice (Figure 6-3D). Together this data shows that UBA1 gene therapy is well tolerated in SMA mice and increasing UBA1 levels in peripheral organs and muscle leads to promising improvements in their weight and motor performance phenotype.
Figure 6-3. Improvement in the weight and motor performance of treated mice following an intraperitoneal injection of AAV9-UBA1 at P1. A-B. Graphs showing average weights (A), righting times (B) of uninjected control (N=10) and SMA mice (N=5) and control (N=7) and SMA (N=5) mice which had received an intraperitoneal injection of AAV9-UBA1 at P1. Data expressed at mean ± SEM. Mice with and without AAV9-UBA1 were compared using a two-tailed unpaired Student’s t-test; * P<0.05. C. Survival curve of uninjected control (N=10) and SMA (N=5) mice and control (N=6) and SMA (N=5) mice which had received an intraperitoneal injection of AAV9-UBA1 at P1. Survival curves were compared using a log-rank (Mantel-Cox) test. D. Representative photos of P8 control and SMA mice treated with an intraperitoneal injection of AAV9-UBA1 at P1.

6.2.4 Intravenous injection via the facial vein is superior at achieving systemic delivery than intraperitoneal injection

Whilst these promising improvements in weights and motor performance in SMA mice showed that increasing UBA1 levels in the peripheral organs and muscle was beneficial, intraperitoneal delivery clearly represented a suboptimal therapy delivery route as it did not lead to robust UBA1 increases in the spinal cord (Figure 6-1B). I therefore next examined whether intravenous injection represented a better delivery method. In particular, I went on to assess the intravascular injection of AAV9 via the facial vein which has previously been shown lead to the widespread transduction of
neurons in the central nervous system, including, importantly for SMA, motor neurons in the spinal cord (Foust et al., 2009).

Injection of Evan’s blue dye via the facial vein in P1 wildtype CD1 pups (Figure 6-4A & B) revealed that the dye was spread quickly around the body via the vascular system resulting in a widespread blue colouration of the injected pup (Figure 6-4C). With intraperitoneal injection, however, the injected dye remained in the intraperitoneal cavity over the same time period (Figure 6-4C). This indicates that intravenous injection via the facial vein is better at achieving rapid systemic expression compared to intraperitoneal delivery.

![Figures 6-4A, 6-4B, 6-4C](image)

**Figure 6-4. A comparison of biodistribution of Evan’s blue dye injected to the intraperitoneal cavity and facial vein in wildtype P1 pups.** A. Photograph of a P1 CD1 mouse pup placed on a transilluminator to increase the visibility of its facial blood vessels. B. Diagram of facial blood vessels of a P1 mouse. Arrow indicates the site of injection into the facial vein. C. Photograph of uninjected (-) P1 CD1 mouse alongside mice which received an intraperitoneal (IP) and intravenous (IV) injection of Evan’s blue dye.

### 6.2.5 Intravenous delivery of AAV9-GFP leads to robust systemic GFP expression and can also target the central nervous system, including the transduction of spinal motor neurons

In wildtype CD1 mice, I next used AAV9-GFP to see if intravenous injection could enable AAV to transduce cells in the CNS as well as examining the temporal expression of genes using AAV9 vectors in different organs. As opposed to the immediate expression seen from transcriptionally competent double stranded DNA template in scAAV vectors, a delay in UBA1 expression from a traditional single
stranded AAV9 vector will be expected (mechanism discussed by (Mccarty et al., 2001)). As illustrated from SMN gene therapy a therapeutic window the rescue of the SMA mouse phenotype is seen with early SMN expression yielding the best therapeutic outcome (Foust et al., 2010). It was therefore important for developing AAV9-UBA1 gene therapy to know when and where expression from AAV9 could be expected.

Following injection of AAV9-GFP via the facial vein at P1, wildtype CD1 pups were culled at P3, P4, P5, P6, P7, P8, P30 and P60 for tissue analysis. At P3, as detected by western blot, high levels of GFP expression were already present in the heart and liver, with moderate amounts in the lungs and muscle (Figure 6-5A & B). GFP expression in these organs (and the kidney) grew stronger overtime from P4-P8 with highest levels still observed in the heart and liver (Figure 6-5A-C). By P30 and P60 both the heart and muscle maintained the high expression levels seen at P8 (Figure 6-5A-C), however for other peripheral organs GFP levels were lower (Figure 6-5A-C). This was particularly obvious in the liver which experienced a ~88% drop in GFP levels from P8 to P30 (Figure 6-5A-C). Whilst absent at P3, by P4 low GFP expression could be detected in the brain and all levels of the spinal cord (Figure 6-5A & B). This expression increased from P4 to P60 indicating that intravenous injection of AAV is capable of eliciting stable gene expression in the CNS.

Immunohistochemistry of the lumbar spinal cord at P8 showed that GFP expression was predominantly located to the dorsal columns with the presence of a number of GFP positive axons extending to lamina X and the ventral horns (Figure 6-5D). Higher magnification confocal microscopy of the ventral horn, revealed GFP positive motor neurons at both P8 and P30 (Figure 6-5E) showing intravenous delivery of AAV can lead to target gene expression in spinal motor neurons.
Figure 6-5. Analysis of the biodistribution and temporal expression of GFP in CD1 wildtype mice following an intravenous injection of AAV9-GFP at P1. A-B. Western blots (A) and graphs showing the western blot quantification (B) of GFP protein.
levels in the brain, heart, liver, lung, kidney, muscle and spinal cord (cervical, thoracic and lumbar levels) of wildtype CD1 mice culled at P3, P4, P5, P6, P7, P8, P30 and P6 following injection of AAV9-GFP via the facial vein at P1 (+) and uninjected controls (-). Dotted lines represent image processing to align lane well order. C. Immunohistochemistry of GFP expression in the heart and liver of P8 uninjected CD1 mice and P8 and P30 mice which received an injection of AAV9-GFP via the facial vein at P1. Scale bar = 250μm. D. GFP immunohistochemistry (green) in the L4-L5 lumbar spinal cord of P8 wildtype mouse which had received an injection of AAV-GFP at P1. Spinal cords counter stained with fluorescent Nissl (blue). Scale bar = 250μm. E. High magnification micrographs of the spinal cord ventral horn from P8 uninjected CD1 mice and P8 and P30 mice which received an injection of AAV9-GFP via the facial vein at P1. Green = GFP immunohistochemistry, blue = fluorescent Nissl. White arrows indicate the presence of GFP in spinal motor neurons. Scale bar = 50μm.

Importantly, AAV9-GFP delivery did not influence Uba1 protein levels in any organ at all time points studied (Figure 6-6 and Appendix 1). This positive control shows that the elevation in UBA1 levels in AAV9-UBA1 treated mice is due to the expression of the virally delivered UBA1 cDNA open reading frame insert and is not just a consequence of injection of AAV9 vectors. AAV9-GFP delivery in wild-type mice did also not alter their body weight or motor performance (Appendix 2).

**Figure 6-6. Analysis of Uba1 protein in CD1 wildtype mice following an intravenous injection of AAV9-GFP at P1.** Western blot of GFP protein levels in the brain, heart, liver, lung, kidney, muscle and spinal cord (cervical, thoracic and lumbar levels) of wildtype CD1 mice culled at P7 following injection of AAV9-GFP via the facial vein at P1 (+) and uninjected controls (-).
6.2.6 Similar biodistribution of GFP in SMA mice and control littermates following injection of AAV9-GFP via the facial vein at P1

Intravenous injection of AAV9-GFP led to the systemic expression of GFP in peripheral organs and the CNS in wildtype CD1 mice. As vascular defects have been reported in SMA mice (Somers et al., 2012), I next wanted to assess if the biodistribution of expression from AAV9 vectors in SMA mice would differ to control littermates. Western blot assessment tissue from P7 control and SMA mice that received a facial vein injection of AAV9-GFP revealed a roughly equal level of GFP expression in the brain, spinal cord, muscle, heart, liver, lung and kidney (Figure 6-7). Similarly, to CD1 wildtype mice, TTG mice showed presence of GFP in the CNS with highest amounts of expression in the heart (Figure 6-7). These data confirm that intravenous delivery of an AAV9 vector is capable of achieving robust systemic target gene expression in peripheral organs and the CNS and shows that the biodistribution in SMA mice is comparable to that of control mice.

Figure 6-7. Similar biodistribution of GFP in SMA and control littermates following injection of AAV9-GFP via the facial vein at P1. Western blots of GFP protein levels in the brain, spinal cord, muscle, heart, liver, lung and kidney of littermate control and SMA mice culled at P7 following injection of AAV9-GFP via the facial vein at P1 (+) and uninjected controls (-).
6.2.7 Intravenous delivery of AAV9-UBA1 is superior to intraperitoneal delivery with stronger UBA1 expression in peripheral organs and ability to transfect spinal motor neurons

As injection of AAV9-GFP via the facial vein resulted in robust systemic and CNS expression in both control and SMA mice, I next wanted to establish if the same was true of AAV9-UBA1. Intravenous delivery of AAV9-UBA1(2.4xE+11 vg titre, 10ul injection volume) AAV9-UBA1 to SMA mice via the facial vein resulted in significantly increased UBA1 protein expression at P7 in the spinal cord, muscle, heart, liver, lung and kidney with no change in the whole brain detectable by western blot (Figure 6-8A & B). Compared to uninjected control levels, these increases were comparable to the changes seen with intraperitoneal delivery in the heart (IP 1888.5% ± 712.9% vs IV 1364.3% ± 84.8%), muscle (IP 216.3% ± 30.5% vs IV 143.1% ± 9.5) and lung (IP 135.4% ± 4.6% vs IV 128.4% ± 6.10%) and greater than intraperitoneal delivery in the liver (IP 264.9% ± 56.2% vs IV 810.9% ± 99.2%), spinal cord (IP 110.5% ± 13.2% vs IV 133.8 ± 12.4%) and kidney (IP 118.2% ± 7.2% vs IV 244.8% ± 36.9%) (Figure 6-1B & 6-8A-B). Immunohistochemical analysis further revealed that at the cellular level, the increase of UBA1 protein in the heart, liver and spinal cord motor neurons in SMA mice treated with AAV9-UBA1 was predominantly nuclear in localisation (Figure 6-8C).

At the mRNA level, RT-qPCR experiments, using primers which could not distinguish between endogenous mouse Uba1 cDNA and virally delivered human UBA1 open reading frame cDNA (Figure 8D), showed a robust increase in total Uba1/UBA1 mRNA expression following intravenous injection of AAV9-UBA1 in the hearts of control littermates (uninjected control 100% ± 73.4% vs control + AAV9-UBA1 20361.3% ± 1397.4%, N=3, P<0.001) (Figure 6-8E). Further RT-qPCR experiments using primer sequences that could differentiate between mouse Uba1 and human UBA1 cDNA (Figure 6-8D), illustrated that the UBA1 increase seen in AAV9-UBA1 injected tissue is exclusively attributable to virally delivered human UBA1 open reading frame cDNA expression as mouse Uba1 mRNA levels remained unchanged in gene therapy treated tissue (Figure 6-8E).
Figure 6-8. Analysis of UBA1 protein and mRNA expression in P7 SMA and control littermates following intravenous injection of AAV9-UBA1 via the facial vein at P1. A-B. Western blots (A) and bar charts showing the western blot quantification (B) of UBA1 protein levels in the brain, spinal cord, *gastrocnemius* muscle, heart, liver, lung and kidney of P7 uninjected SMA mice and P7 SMA mice which had received an intravenous...
injection of AAV9-UBA1 at P1. UBA1 levels are expressed as a percentage of uninjected SMA levels and are shown as mean ± SEM. N=3 for each treatment group. *P<0.05, **P<0.01, ***P<0.005, ns = not significant; unpaired, two-tailed Student’s t-test. C. Immunohistochemistry of UBA1 (green) in the heart, liver and spinal cord ventral horn of P7 uninjected SMA mice and SMA mice which received an injection of AAV9-UBA1 via the facial vein at P1. Hearts and livers co-labelled with DAPI and spinal cord fluorescent Nissl (blue). Scale bar = 50μm. D. PCR products using primers that detect both mouse Uba1 and human UBA1 cDNA (top band, primers that only detect mouse Uba1 cDNA (middle band) and primers that only detect human UBA1 cDNA. E. RT-qPCR quantification of mouse and human Uba1/UBA1 mRNA expression in hearts of P7 uninjected control mice and P7 control mice which had received an intravenous injection of AAV9-UBA1 at P1 using the primers shown in panel E. Data are expressed as a percentage of uninjected control levels and are shown as mean ± SEM. N=3 per treatment group. Each sample was amplified in triplicate and average taken before normalising to expression of housekeeping genes. ****P<0.001, ns = not significant; unpaired, two-tailed Student’s t-test.

6.2.8 Intravenous injection of AAV-UBA1 is safe in control mice in the long-term

As intravenous delivery of AAV9-UBA1 led to higher levels of UBA1 overexpression than intraperitoneal injection I also wanted to establish the safety of this delivery route in control mice. AAV9-UBA1 intravenous delivery did not adversely affect the weight of control littermates from P3 to P9 (Figure 9A) or in the long-term at P30 or P60 (Appendix 3). The motor performance of control mice was also unaffected (Figure 9B). Control mice receiving AAV9-UBA1 via the facial vein at P1 also had a normal overt healthy appearance both as pups (Figure 9C) and as adults (Appendix 3). To investigate any more subtle health effects UBA1 overexpression may be having in vivo, a haematology and blood serum biochemistry analysis was performed on P30 AAV9-UBA1 treated mice and age matched uninjected controls. In all 19 haematology and 11 serum biochemistry parameters measured, no significant alterations were detected (Appendix 4 and 5). The absence of any significant changes to blood cell parameters and biochemistry levels further indicates that UBA1 gene therapy is safe and well tolerated in mice.
6.2.9 Intravenous delivery of AAV9-UBA1 leads to improvements in the weight and motor performance of SMA mice

I next wanted to assess the impact of intravenously delivered AAV9-UBA1 on the phenotype of SMA mice. SMA mice injected with AAV9-UBA1 via the facial vein at P1 had significantly increased weights at late-symptomatic stages compared to uninjected controls. For example at P9, uninjected SMA mice weighed 1.76g ± 0.03g (N=25) compared to 2.48g ± 0.19g (N=4) for SMA mice which had received an intravenous injection of AAV9-UBA1 at P1 (P<0.005) (Figure 6-9A). The righting times of SMA mice following UBA1 gene therapy were strikingly improved to near control levels from P4 onwards (Figure 6-9B). At P9 for example, uninjected SMA mice took 9.7s ± 1.2s to right themselves whereas SMA AAV9-UBA1 treated mice only took 2.6s ± 0.4s (P<0.05). At late-symptomatic stages, SMA mice treated with intravenous UBA1 gene therapy also had slightly healthier appearance (Figure 6-9C). Together this data shows that intravenous delivery of AAV9-UBA1 has a beneficial impact on the SMA mouse disease phenotype, leading to improvements in weight and motor performance particularly at late-symptomatic ages.
6.2.10 Indication that AAV9-UBA1 leads to improvements in NMJ pathology in SMA mice

As UBA1 gene therapy led to the robust improvement in motor performance of SMA mice it was important to assess the impact of increased UBA1 levels on NMJ pathology. As shown previously, SMA mice have a reduced number of axonal inputs.
to the NMJ in the *levator auris longus* (LAL) muscle (Wishart et al., 2014) (Figure 10A). This is reflected in a larger percentage of mono-innervated endplates as opposed to poly-innervated endplates in SMA mice (Figure 6-10B). In P9 LAL muscles of mice treated with intravenous AAV9-UBA1 at P1, this denervation appeared ameliorated in UBA1 gene therapy treated SMA mice with NMJ axonal inputs returning to littermate control levels (Figure 6-10A&B). This data indicates that increasing UBA1 levels may lead to improvement in SMA NMJ pathology. However, due to the small sample size and large inter-animal variability, further analysis is necessary to increase the N numbers required for achieving robust statistical significance.

**Figure 6-10.** NMJ analysis of P9 SMA mice and control littermates following an intravenous injection of AAV9-UBA1 at P1. A-C. Bar chart showing the average axonal input per NMJ (A), the percentage of mono-innervated or poly-innervated NMJ endplates (B) in the rostral band of the *levator auris longus* (LAL) muscle of P9 SMA mice and control littermates following an intravenous injection of AAV9-UBA1 at P1. Data shown as mean ± SEM; average of 50 NMJs chosen at random per muscle. N=4 for each treatment group. Changes not significant; unpaired, two-tailed Student’s t-test.
6.2.11 AAV9-UBA1 gene therapy corrects downstream UPS molecular perturbations in SMA mice

I next wanted to examine the consequences of increasing UBA1 in vivo on the downstream UPS molecular perturbations previously identified in the neuromuscular system of late-symptomatic SMA mice (Wishart et al., 2014). Heart tissue was chosen to be investigated by western blot and proteomic analysis as Uba1 protein reduction in organs of Taiwanese mice is greatest in the heart tissue (Chapter 3, Figure 3-1) and as AAV9-UBA1 gene therapy leads to the greatest increase in UBA1 levels in the heart (Figure 6-8). Western blot analysis revealed a significant reduction of β-catenin levels in P7 uninjected SMA mouse hearts and P7 SMA mouse hearts which had received an intravenous injection of AAV9-UBA1 at P1 (SMA uninjected 100% ± 6.9% vs SMA+AAV9-UBA1 66.8% ± 6.5%, P<0.05) whilst mono-ubiquitin (SMA uninjected 100% ± 8.9% vs SMA+AAV9-UBA1 168.5% ± 2.4%, P<0.005), di-ubiquitin (SMA uninjected 100% ± 9.4% vs SMA+AAV9-UBA1 217.4% ± 15.1%, P<0.01) and poly-ubiquitin (SMA uninjected 100% ± 8.1% vs SMA+AAV9-UBA1 145.7% ± 5.4%, P<0.01) levels were all significantly increased (Figure 6-11). Uchl1 and tri-ubiquitin protein levels remained unchanged (Figure 6-11). Importantly, this data shows that the virally delivered human UBA1 gene is functional in mice in vivo and that increasing UBA1 levels can correct the downstream UPS perturbations of increased β-catenin and reduced mono- and poly-ubiquitin levels in SMA mice.
Figure 6-11. Western blot analysis of UPS proteins of interest in P7 SMA following an intravenous injection of AAV9-UBA1 at P1. A. Western blots of β-catenin, Uchl1, polyubiquitin, tri-ubiquitin, di-ubiquitin, monoubiquitin protein levels in the heart of P7 SMA mice which had received an injection of AAV9-UBA1 via the facial vein at P1 (+) and uninjected SMA controls (-). B-G. Bar charts showing the quantification of the western blots measuring β-catenin (B), Uchl1 (C), monoubiquitin (D), di-ubiquitin (E), tri-ubiquitin (F), polyubiquitin (G) protein levels in the heart of uninjected P7 SMA mice and P7 SMA mice following an injection of AAV9-UBA1 via the facial vein at P1. Protein levels are expressed as a percentage of uninjected SMA levels and are shown as mean ± SE. N=3 for each treatment group. *P<0.05, **P<0.01, ***P<0.005, ns = not significant; unpaired, two-tailed Student’s t-test.

6.2.12 Proteomic analysis of control AAV9-UBA1 heart tissue highlights functional roles of UBA1 in vivo and the altered expression of proteins that are relevant to SMA

To further explore the molecular consequences of increasing UBA1 protein levels in vivo, label free proteomic analysis was conducted on the hearts of P7 control mice which had received an injection of AAV9-UBA1 via the facial vein at P1. Protein
level changes were compared against hearts of P7 uninjected control mice. To identify proteins altered as a consequence of increased UBA1 levels, as opposed to proteins changed due to the introduction of AAV9, proteomic analysis was also conducted on control P7 mouse hearts which had received an intravenous injection of AAV9-GFP at P1.

Analysis of mass-spectrometry data using Progenesis QI Proteomics revealed 788 proteins present in all experimental groups (Figure 6-12B). Filtering was applied to find proteins identified by ≥2 unique peptides and with a significant \( P \leq 0.05 \) 20% fold change between groups. A total of 198 proteins were changed in AAV9-UBA1 treated hearts compared to uninjected controls (Figure 6-12B). Of these proteins, 47 were also changed in AAV9-GFP treated tissue compared to uninjected controls (Figure 6-12A & B, Appendix 8). These 47 proteins were subsequently filtered out as being changed due to the response to AAV9 viral vectors (Figure 6-12B). Indeed, top canonical pathway \textit{in silico} analysis of these 47 proteins by Ingenuity Pathway Analysis (IPA) software identified changes in acute phase response signalling and interferon signalling (Appendix 11), indicating an acute immune response following AAV9 delivery. This is consistent with the published literature that AAV mediated gene therapy can trigger humoral and cellular immune responses (reviewed by (Sun et al., 2003)).

A total of 151 protein changes (59 upregulated and 92 down regulated) could therefore be attributable to increased UBA1 levels in the heart of P7 control mice (Figure 6-12A & B, Appendix 6 & 7). Reassuringly, UBA1 was the detected as having the greatest upregulation (8.71 fold increase) in AAV9-UBA1 treated tissue compared to controls (Figure 6-13B & Appendix 6). β-catenin levels were also detected as being down regulated (-1.33 fold change) further validating the reliability of proteomic results (Appendix 7). Proteins changed exclusively in AAV9-GFP tissue are shown in Appendix 9 & 10, with IPA top canonical pathway analysis shown in Appendix 12.
Figure 6-12. Proteomic analysis to identify proteins changed in response to increasing UBA1 levels in vivo. A. Venn diagram of proteomic data showing that compared to uninjected P7 control mouse hearts, 151 proteins were exclusively changed in P7 control hearts treated with AAV9-UBA1 intravenous injection at P1, 17 proteins were exclusively changed P7 control hearts treated with AAV9-GFP intravenous injection at P1 and 47 proteins were changed in both AAV9-UBA1 and AAV9-GFP treated groups. B. Scatterplots showing fold change of proteins in the heart of AAV9-UBA1 treated mice compared to controls. Right scatterplot shows fold changes of 788 proteins present in uninjected, AAV9-UBA1 and AAV9-GFP groups identified by Progenesis QI Proteomics.
software following label-free mass spectrometry. Middle scatter plot shows fold changes of the 198 proteins changed in AAV9-UBA1 treated hearts compared to uninjected controls after filtering was applied to find proteins identified by ≥2 unique peptides and with a significant (P ≤ 0.05) 20% fold change between groups. Right scatterplot shows fold changes of the 151 proteins changed only in AAV9-UBA1 treated hearts compared to uninjected controls following the elimination of the 47 proteins that were also changed >20% in AAV9-GFP treated hearts. Arrow shows the identification of UBA1. Red lines indicate the 20% cut off threshold for being up-regulated (1.2 fold change) or down-regulated (-1.2 fold change) in AAV9-UBA1 treated tissue compared to uninjected controls.

Bioinformatic analysis of the 151 proteins uniquely up-regulated in AAV9-UBA1 treated hearts by Ingenuity Pathway Analysis (IPA) software was then conducted to better understand the functional consequence of increasing UBA1 levels in vivo. Canonical pathway analysis revealed that proteins changed due to increased UBA1 levels are mainly clustered in to pathways associated with alterations in mitochondrial function and actin cytoskeletal signalling (Table 6-1). Interestingly, the canonical pathway of ‘protein ubiquitination’ was not highlighted indicating that UBA1 gene therapy does not lead to widespread disruption of UPS homeostasis. Indeed out of the 151 proteins, only 2 that are directly involved in the UPS were significantly changed: ubiquitin carboxyl-terminal hydrolase 28 (Usp28, -1.31 fold change) and proteasome subunit 1(Psmb1, 1.37 fold change) (Appendix 6 & 7). The surprising enrichment of proteins related to ‘neurological disease’ and ‘psychological disorders’ enriched in heart tissue following UBA1 gene therapy (Table 6-2) may indicate that UBA1 is of particular importance to nervous system function. IPA analysis of the ‘molecular and cellular functions’ of proteins changed due to increased UBA1 levels suggests that at the cellular level, UBA1 plays important roles in cell morphology, development, growth and metabolism (Table 6-3).
Table 6-1. Top canonical pathways identified by Ingenuity Pathway Analysis (IPA) proteomic analysis of proteins with a >20% change in AAV9-UBA1 P7 control hearts.

<table>
<thead>
<tr>
<th>Name</th>
<th>P-value</th>
<th>Overlap</th>
<th>Individual proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial dysfunction</td>
<td>1.49E-12</td>
<td>8.8%</td>
<td>HSD17B10, SDHA, NDUFV1, SDHB, NDUF68, ATP5A1, CPT1B, ACO2, NDUF33, NDUF31, UQCRCL2,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NDUF32, UQCRFS1, OGDH, AIFM1</td>
</tr>
<tr>
<td>Fatty acid oxidation I</td>
<td>1.54E-09</td>
<td>23.3%</td>
<td>HSD17B10, HADH, ACAA2, ACAD, ACADL1, HADHA, HADH</td>
</tr>
<tr>
<td>Actin cytoskeleton signaling</td>
<td>5.49E-09</td>
<td>6.0%</td>
<td>MYH10, MYH9, MYH9, CFL1, ACTN3, MYL3, TNIK, TTN, TTN2, FLNA, ARPC4, MYL12A</td>
</tr>
<tr>
<td>Oxidative phosphorylation</td>
<td>5.85E-09</td>
<td>9.2%</td>
<td>SDHA, NDUFV1, SDHB, NDUF31, NDUF36, ATP5A1, UQCRCL2, NDUF32, UQCRFS1, NDUF33</td>
</tr>
<tr>
<td>Glutaryl-coA degradation</td>
<td>7.71E-09</td>
<td>45.5%</td>
<td>HSD17B10, HADH, CAT1, HADHA, HADH</td>
</tr>
</tbody>
</table>

Table 6-2. Diseases and disorders identified by Ingenuity Pathway Analysis (IPA) proteomic analysis of proteins with a >20% change in AAV9-UBA1 P7 control hearts.

<table>
<thead>
<tr>
<th>Diseases and disorders</th>
<th>P-value</th>
<th>Number of molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hereditary disorder</td>
<td>7.14E-03 - 2.01E-17</td>
<td>78</td>
</tr>
<tr>
<td>Neurological disease</td>
<td>7.14E-03 - 2.01E-17</td>
<td>66</td>
</tr>
<tr>
<td>Psychological disorders</td>
<td>4.69E-03 - 2.01E-17</td>
<td>42</td>
</tr>
<tr>
<td>Skeletal and muscular disorders</td>
<td>7.14E-03 - 2.01E-17</td>
<td>62</td>
</tr>
<tr>
<td>Developmental disorder</td>
<td>7.14E-03 - 1.2E-15</td>
<td>59</td>
</tr>
</tbody>
</table>
Table 6-3. Molecular and cellular functions identified by Ingenuity Pathway Analysis (IPA) proteomic analysis of proteins with a >20% change in AAV9-UBA1 P7 control hearts.

<table>
<thead>
<tr>
<th>Molecular and cellular functions</th>
<th>P-value</th>
<th>Number of molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>7.14E-03 - 1.01E-15</td>
<td>47</td>
</tr>
<tr>
<td>Cellular development</td>
<td>7.14E-03 - 1.56E-10</td>
<td>16</td>
</tr>
<tr>
<td>Cellular growth and proliferation</td>
<td>7.14E-03 - 1.56E-10</td>
<td>68</td>
</tr>
<tr>
<td>Energy production</td>
<td>7.14E-03 - 1.83E-10</td>
<td>19</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>7.14E-03 - 1.83E-10</td>
<td>33</td>
</tr>
</tbody>
</table>

Interestingly, increasing UBA1 levels in vivo led to the altered expression of proteins that are relevant to SMA (Table 6-4). Plastin-3 (PLS3), an actin binding protein which plays a role in SMA axonal actin dynamics (Oprea et al., 2008, Ackermann et al., 2013) and acts as a protective modifier in some SMA patients (Oprea et al., 2008, Stratigopoulos et al., 2010, Bernal et al., 2011) had a 1.6 fold increase in AAV9-UBA1 hearts (Appendix 6). Notably, there were also increases in the SMN splicing enhancer Transformer-2 protein homolog beta (Tra2b), the SMN nuclear import protein Importin subunit beta-1 (Kpnb1) and the SMN binding component of SNRNPs Small nuclear ribonucleoprotein Sm D3 (Snrdp3) (Appendix 6). Other SMN binding proteins Annexin A2 (Anxa2), Myb-binding protein 1A (Mybbp1a) Eukaryotic initiation factor 4A-I (Eif4a1) were also increased in AAV9-UBA1 treated hearts (Appendix 6). There was also some overlap between proteins changed due to altered UBA1 expression and proteins that have shown to be altered in SMA proteomic screens (Wishart et al., 2014, Murray et al., 2010b, Mutsaers et al., 2011, Wishart et al., 2010, Mutsaers et al., 2013, Aghamaleky Sarvestany et al., 2014) as well as proteins that have altered phosphorylation in SMA (Nolle et al., 2011) or are predicted to have altered mRNA splicing (Zhang et al., 2008).
Table 6-4. Proteins with a >20% change in AAV9-UBA1 P7 control hearts compared to uninjected controls that are relevant to SMA.

<table>
<thead>
<tr>
<th>Role in SMA</th>
<th>Individual proteins</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protective modifier in some SMA patients</td>
<td>Pls3</td>
<td>(Oprea et al., 2008, Stratigopoulos et al., 2010, Bernal et al., 2011)</td>
</tr>
<tr>
<td>SMA axonal actin dynamics dysfunction</td>
<td>Pls3</td>
<td>(Oprea et al., 2008, Ackermann et al., 2013)</td>
</tr>
<tr>
<td>SMN splicing enhancer (SMN binding)</td>
<td>Tra2b</td>
<td>(Hofmann et al., 2000, Hofmann and Wirth, 2002)</td>
</tr>
<tr>
<td>SMN nuclear import (SMN binding)</td>
<td>Kpnb1</td>
<td>(Narayanan et al., 2002)</td>
</tr>
<tr>
<td>Component of snRNPs (SMN binding)</td>
<td>Snrpd3</td>
<td>(Buhler et al., 1999, Pellizzoni et al., 1999, Friesen and Dreyfuss, 2000)</td>
</tr>
<tr>
<td>Other SMN binding proteins</td>
<td>Anxa2</td>
<td>(Shafey et al., 2010, Fuller and Morris, 2010)</td>
</tr>
<tr>
<td>Anxb1a</td>
<td></td>
<td>(Fuller and Morris, 2010, Fuller et al., 2010)</td>
</tr>
<tr>
<td>Eif4a1</td>
<td></td>
<td>(Shafey et al., 2010)</td>
</tr>
<tr>
<td>Altered expression in SMA mouse tissue as identified by proteomic screens</td>
<td>Uba1, Cinnb1, Atp1b1, Pgam1, Atp6v1a, Dbi, Eno1, Tpt1, Cs, Ppa1</td>
<td>(Wishart et al., 2014)</td>
</tr>
<tr>
<td>Anxa2, Col15a1, Lamb1</td>
<td></td>
<td>(Murray et al., 2010b)</td>
</tr>
<tr>
<td>Anxa2, Fina, Eif4a1, Eef2, Tpt1, Mdh2, Aco2, Cs, Srl, Actn2, Myom1, Casq2, Myom2</td>
<td></td>
<td>(Mutsaers et al., 2011)</td>
</tr>
<tr>
<td>Cct2, Pgam1, Dbi</td>
<td></td>
<td>(Wishart et al., 2010)</td>
</tr>
<tr>
<td>Anxa2, Actn2</td>
<td></td>
<td>(Mutsaers et al., 2013)</td>
</tr>
<tr>
<td>Uba1, Anxa2, Pls3, Fina, Eif4a1, Gsn, Rnh1, Clic1, Ywhaz, Eef2, Plec, Pgam1, Kpnb1, Cll1, Cct2, Tcp1, Hadha, Atpsa1, Lamc1, Dsp, Igf2r</td>
<td></td>
<td>(Aghamaley Sarvestany et al., 2014)</td>
</tr>
<tr>
<td>Splicing predicted to be affected in SMA</td>
<td>Col15a1, Tln2, Acs1, Ktn1, Psmb1</td>
<td>(Zhang et al., 2008)</td>
</tr>
<tr>
<td>Altered phosphorylation in SMA</td>
<td>Clt1</td>
<td>(Nolle et al., 2011)</td>
</tr>
</tbody>
</table>

To validate these findings in SMA mice, I next measured the expression of Plastin-3 and Tra2b in hearts from P7 SMA mice treated with AAV9-UBA1 at P1 compared to uninjected SMA controls by western blot. Plastin-3 levels were increased by 204% ± 52% (P<0.05) whereas Tra2b levels were increased by 69% ± 12% (P<0.05) in the hearts of AAV9-UBA1 injected SMA mice (Figure 6-13). These increases in Plastin-3 and Tra2b levels in SMA mice following UBA1 gene therapy therefore validate proteomic results from control littermates showing that increased UBA1 levels in vivo causes the upregulation of proteins relevant to SMA.
Figure 6-13. Western blot validation of proteomic analysis in AAV9-UBA1 SMA mouse hearts: increased expression of Plastin-3 and Tra2b. A. Western blots showing Plastin-3 (top panel) and Tra2b (middle panel) protein levels in the heart of P7 uninjected SMA mice (−) and P7 SMA mice which had received an intravenous injection of AAV9-UBA1 at P1 (+). Bottom panel shows α-tubulin loading. B-C. Bar charts showing the quantification of Plastin-3 (B) and Tra2b (C) protein levels in the heart of P7 uninjected SMA mice and P7 SMA mice which had received an intraperitoneal injection of AAV9-UBA1 at P1. Protein levels are expressed as a percentage of uninjected SMA levels and are shown as mean ± SEM. N=3 for each treatment group. *P<0.05; unpaired, two-tailed Student’s t-test.

6.2.13 UBA1 gene therapy leads to an increase in SMN protein and FL-SMN mRNA expression in the hearts of SMA mice

As AAV9-UBA1 led to an increase in several proteins of interest to SMA, including many SMN binding partners, I next wanted to see if the expression of SMN itself was altered following UBA1 gene therapy. As expected, western blot analysis revealed a significant reduction in SMN and UBA1 levels in the hearts of late-symptomatic P7 SMA mice compared to control littermates (Figure 6-14A & C). Intravenous injection of AAV9-UBA1 at P1 led to similar increases in the level of UBA1 in P7 control mice (1091% ± 207% increase, P<0.01) and P7 SMA mice (1264% ± 84% increase, P<0.01) (Figure 6-14A & C). SMN protein levels in both control mice (uninjected control 100% ± 5.7% vs AAV9-UBA1 control 128.7% ± 7.5%) and SMA mice (uninjected SMA 4.4% ± 1.9% vs AAV9-UBA1 SMA 21.8% ± 2.9%) (Figure 6-14A & B) treated with UBA1 gene therapy. No change in UBA1 or SMN levels were detected in control and SMA mice that had received an intravenous injection of AAV9-GFP (Appendix 13). RT-qPCR quantification of Δ7-SMN and FL-SMN mRNA expression in hearts of P7 uninjected SMA mice and P7 SMA mice which had received an intravenous injection of AAV9-UBA1 at P1.
revealed that while Δ7-SMN cDNA levels were not significantly changed (Figure 6-14 D&E) FL-SMN were increased following UBA1 overexpression (uninjected control 100% ± 54.1% vs AAV9-UBA1 control 300.7% ± 21.3%) (Figure 6-14 D&E). Together, this data shows that increasing UBA1 levels can lead to increased expression of FL-SMN mRNA and SMN protein in vivo.

Figure 6-14. Analysis of SMN protein and mRNA expression in P7 SMA and control littermates following intravenous injection of AAV9-UBA1 via the facial vein at P1. A. Western blots showing UBA1 (top panel) and SMN (middle panel) protein levels in the heart of P7 uninjected control and SMA mice (-) and P7 control and SMA mice which had received an intravenous injection of AAV9-UBA1 at P1(+). Bottom panel shows α-tubulin loading. B-C. Bar charts showing the quantification of SMN (B) and UBA1 (C) protein levels in the heart of P7 uninjected control and SMA mice and P7 control and SMA mice which had received an intravenous injection of AAV9-UBA1 at P1. Protein levels are expressed as a percentage of uninjected control levels and are shown as mean ± SEM. N=3 for each treatment group. **P<0.01, ***P<0.005; unpaired, two-tailed Student’s t-test. D. PCR products using primers that detect full-length (FL) SMN cDNA (top band) and primers that detect Δ7-SMN cDNA (bottom band). E. RT-qPCR quantification of Δ7-SMN and FL-SMN mRNA expression in hearts of P7 uninjected SMA mice and P7 SMA mice which had received an intravenous injection of AAV9-UBA1 at P1 using the primers shown in panel E. Data are expressed as a percentage of uninjected SMA levels and are shown as mean ± SEM. N=3 per treatment group. Each sample was amplified in triplicate and average taken
before normalising to expression of housekeeping genes. *P<0.05, ns = not significant; unpaired, two-tailed Student’s t-test.

6.3 Discussion

Previous findings have shown that reduced Uba1 activity directly contributes to SMA pathogenesis (Wishart et al., 2014) suggesting that increasing UBA1 levels may be of therapeutic benefit. In this chapter I have characterised the impact of increasing UBA1 levels using gene therapy as a novel therapeutic strategy in SMA mice. Initial studies using an intraperitoneal delivery route established that AAV9-UBA1 gene therapy is capable of robustly increasing UBA1 levels in the peripheral organs and muscle of SMA mice in vivo. This was an encouraging finding, especially considering the multisystem reduction of Uba1 in all major organs in the Taiwanese mouse model (Chapter 3). The systemic transduction of UBA1 in SMA mice using AAV9, particularly to the heart, is consistent with previous extravascular biodistribution studies and the known serotype tropism of AAV9 (Inagaki et al., 2006). Crucially, the absence of overt adverse effects over a long-term period in control littermate mice following intraperitoneal AAV9-UBA1 injection at P1 indicated that increasing levels of the main E1 ubiquitin activating enzyme in vivo is safe and well tolerated. In SMA mice, improvements in the weight and motor performance excitingly suggested that increasing UBA1 levels in peripheral organs and muscle may lead to promising therapeutic outcomes.

To optimise the impact of UBA1 gene therapy, I next assessed if intravenous injection represented a better delivery method. Intravenous injection of Evan’s blue dye via the facial vein in P1 wildtype mice showed that better at achieving rapid systemic expression compared to intraperitoneal delivery. These findings were confirmed with intravenous delivery of AAV9-GFP, which in wildtype mice led to robust systemic GFP expression and targeting the central nervous system including, and in line with previously published reports (Foust et al., 2009), the transduction of spinal motor neurons. Intravenous injection of AA9-UBA1 also led to comparable or improved expression of UBA1 levels in peripheral organs compared with intraperitoneal delivery, as well as leading to increased UBA1 in the spinal cord,
including spinal motor neurons, of SMA mice. Importantly, no change in UBA1 levels was seen with AAV9-GFP treated mice indicating that the increases in UBA1 seen are due to virally delivered UBA1 cDNA open reading frame insert and is not just a consequence of injection of AAV9 vectors. This was confirmed with RT-qPCR analysis showing that increases in UBA1 mRNA expression were exclusively attributable to virally delivered human UBA1 open reading frame cDNA expression as mouse Uba1 mRNA levels remained unchanged in gene therapy treated tissue.

Immunohistochemical analysis revealed that the increase in UBA1 levels was predominantly nuclear which was as to be expected considering the virally delivered cDNA UBA1 reading frame represents the full length UBA1a splice variant which has a nuclear localisation signal (Stephen et al., 1997). Previous work has shown that motor neurons of SMA mice at mid- and late-symptomatic stages display a marked a marked redistribution of Uba1 from the cytoplasm to the nucleus (Wishart et al., 2014). To what extent it is important to restore the normal localisation of UBA1 in motor neurons for therapeutic effect is not known. As increasing nuclear UBA1 levels in vivo seems to result in beneficial effects in SMA mice it may be that the altered localisation of UBA1 in motor neurons represents a compensatory mechanism to try and increase UBA1 dependent ubiquitination events in the nucleus. As UBA1 is known to function in DNA repair (Nouspikel and Hanawalt, 2006, Moudry et al., 2012), this raises the possibility that the altered localisation of UBA1 in SMA motor neurons could therefore help with the DNA fragmentation known to occur in the SMA mouse tissue (Fayzullina and Martin, 2014). Further analysis using markers of DNA fragmentation and repair could be used to test this hypothesis in the AAV9-UBA1 treated mice. Future work using subcellular fractionation could also establish whether the UBA1 cytoplasmic fraction is also increased using the full length UBA1 vector in this study.

Intravenous delivery of AAV9-UBA1 led to improvements in the weights of SMA mice at late-symptomatic stages as well as a robust improvement in motor performance to near control levels. This improvement in motor performance was reflected in the indications of improvements in SMA NMJ pathology although due to the small sample size and large variability, further analysis is necessary to increase
the N numbers required for achieving robust statistical significance. It will also be interesting to see if these improvements of denervation at the NMJ are reflected at the level of the spinal cord, with further work to be done to assess if UBA1 gene therapy can ameliorate motor neuron loss in SMA mice. As Uba1 loss is a consistent feature of three independent SMA mouse models it will be also essential to assess the impact of increasing UBA1 levels on muscle pathology such as the reduction in muscle fibre diameter seen in SMA mice. Interestingly, *Drosophila* with *Uba1* hypomorphic mutations display motor impairments (Liu and Pfleger, 2013) and *C. elegans Uba1* loss-of-function mutants show, amongst other developmental alterations, a male-specific progressive tail paralysis (Kulkarni and Smith, 2008) indicating that sufficient levels of Uba1 are particularly important for normal neuromuscular function. Moreover, considering the multisystem reduction in Uba1 levels in Taiwanese mice and as AAV-UBA1 intravenous injection led to the greatest increase in UBA1 levels in peripheral organs, it would also be interesting to see the impact of UBA1 gene therapy outside of the neuromuscular system on the morphology or function of SMA mouse organs such as the heart or liver. Future work should also crucially assess the impact of intravenous delivered AAV9-UBA1 on the survival of SMA mice.

Western blot analysis of AAV9-UBA1 treated SMA mouse hearts showed that the virally delivered human UBA1 gene is functional in mice *in vivo* and that increasing UBA1 levels can correct the downstream UPS perturbations by reducing β-catenin and increasing mono- and poly-ubiquitin levels previously identified in the neuromuscular system of SMA mice (Wishart et al., 2014). The correction of downstream UPS perturbations could be contributing to the improved motor performance of UBA1 gene therapy treated mice as β-catenin inhibition with quercetin as be shown to improve the SMA mouse neuromuscular phenotype (Wishart et al., 2014). However, in the heart of SMA mice where Uba1 levels are robustly decreased (Chapter 3), unlike in the neuromuscular system β-catenin levels don’t accumulate (Wishart et al., 2014). The reduction in β-catenin heart levels following UBA1 gene therapy shows that heart β-catenin are in fact responsive to changes in UBA1 levels and further indicates that peripheral organs of SMA mice have different molecular characteristics that mean they are less susceptible to
downstream UPS alterations caused reduced Uba1 levels than the neuromuscular system. The increase in monoubiquitin levels in AAV9-UBA1 treated SMA mouse hearts is consistent with the finding of reduced monoubiquitin levels in cells were Uba1 activity in inhibited (Chapter 5). However, unlike in Uba1 inhibited cells where the downstream DUB Uch1 was increased, no converse decrease in Uch1 levels were detected in AAV9-UBA1 treated tissue. The increase in polyubiquitin levels in UBA1 gene therapy hearts indicates a higher level of substrate ubiquitination and therefore perhaps a high DUB activity is still required to contribute to the increased ubiquitination cycle activity. An increase in ubiquitin conjugation following an increase in Uba1 activity has also been seen in yeast Uba1 temperature sensitive mutants (Ghaboosi and Deshaies, 2007). It would be, therefore, interesting to assess the impact of increased conjugation on the downstream proteasome activity in AAV9-UBA1 treated SMA mice. Importantly, however, proteomic analysis of AAV9-UBA1 control mouse hearts did not highlight the canonical pathway of ‘protein ubiquitination’ as being significantly changed, indicating that UBA1 gene therapy does not lead to the widespread disruption of UPS proteins.

Proteomic analysis of AAV9-UBA1 control mouse hearts also highlighted the role of UBA1 in mitochondria and actin cytoskeletal signalling. Alterations in mitochondrial proteins are perhaps to be expected considering the importance of ubiquitination in mitochondrial function (Livnat-Levanon and Glickman, 2011) with UBA1 itself known to be important for the clearance of mitochondria (Chang et al., 2013). The increase in proteins involved in the canonical pathway of actin cytoskeletal signalling following UBA1 upregulation was an intriguing finding, especially considering the reported axonal and growth cone actin dynamics dysfunction in cultured SMA mouse motor neurons (Rossoll et al., 2003, Nolle et al., 2011). Not much is known about the function of UBA1 in this non-degradative role, although the requirement of ubiquitination in localized actin assembly and regulation of protein anterograde transport from the trans-Golgi network has recently been elucidated (Yuan et al., 2014).
The surprising enrichment of proteins related to ‘neurological disease’ and ‘psychological disorders’ enriched in heart tissue following UBA1 gene therapy also indicates UBA1 is of particular importance to nervous system function. Indeed, in the axons of γ neurons of mushroom bodies (Watts et al., 2003) and dendrites of peripheral sensory neurons (Kuo et al., 2005) of Drosophila loss-of-function Uba1 mutations have been shown to strikingly inhibit developmental pruning illustrating that Uba1 is important molecular component in axon remodelling processes. Interestingly, UBA1 is upregulated in the synapses of Wld⁺ mice, a model in which axons and synapses are protected from Wallerian degeneration (Wishart et al., 2007). UBA1 levels are also increased in the cerebellum of Wld⁺ mice where enhanced expression was restricted to the nucleus rather than the cytoplasm (Wishart et al., 2008). This is in potential agreement with the theory that Uba1 distribution changes seen in SMA motor neurons represent as a compensatory neuroprotective change and suggests it may therefore beneficial that nuclear UBA1 levels are increased following gene therapy in this study. Changes in the cell cycle are also noted in Wld⁺ mice (Wishart et al., 2008) and considering the well documented role of UBA1 in cell cycle progression (Grenfell et al., 1994, Sudha et al., 1995, Jin et al., 2007, Lee et al., 2008, Sugaya et al., 2014) it would be worth checking if any such changes are also present following UBA1 gene therapy. This is particularly pertinent as gene mutations or alterations in UBA1 expression or has been associated with cell proliferation (Lee et al., 2008) and cancer (Rogers et al., 2013, Xu et al., 2010) with pharmacological inhibition of Uba1 delaying tumour growth in a mouse model of leukaemia (Xu et al., 2010). Reassuringly, no signs of tumour formation were noted in either SMA mice up to P9 or control mice over a two month period and additionally no changes in cell cycle proteins were detected in the proteomic screen as a consequence of UBA1 overexpression. Previous work has also shown that Uba1 overexpression had no effect on the proliferation rate in the B16-F0 mouse skin melanoma cell line with syngeneic transplantation of Uba1 overexpressing B16-F0 cells not significantly affecting the number of melanoma colonies in the lungs elicited in wildtype mice (Rogers et al., 2013).

Proteomic analysis of AAV9-UBA1 control mouse hearts also highlighted alterations in proteins are relevant to SMA particularly the upregulation of numerous SMN
binding partners. Of particular note, were increased expression of the \textit{SMN} splicing enhancer Transformer-2 protein homolog beta (Tra2b), the \textit{SMN} nuclear import protein Importin subunit beta-1 (Kpnb1), the \textit{SMN} binding component of \textit{SNRNPs} Small nuclear ribonucleoprotein Sm D3 (Snrpd3) and Plastin-3 (PLS3) an actin binding protein which plays a role in SMA axonal actin dynamics (Oprea et al., 2008, Ackermann et al., 2013) and acts as a protective modifier in some SMA patients (Oprea et al., 2008, Stratigopoulos et al., 2010, Bernal et al., 2011). Further examination of the expression of \textit{SMN} itself in SMA mice and control littermates excitingly revealed that increasing \textit{UBA1} levels using gene therapy approaches can lead to increased expression of FL-SMN mRNA and \textit{SMN} protein \textit{in vivo}. The increase in \textit{SMN} and \textit{SMN}-binding proteins which are known to modify the disease phenotype may therefore be contributing to the therapeutic effects seen with \textit{UBA1} gene therapy treated SMA mice. Whilst it is known that the UPS is the main system that contributes to \textit{SMN} degradation (Burnett, 2009; Chang, 2004) further work can now to be done to fully understand this reciprocal regulation of \textit{UBA1} and \textit{SMN}. For example, does the increase in FL-SMN but not Δ7-SMN mRNA expression in AAV9-%textit{UBA1} treated hearts mean that \textit{SMN2} splicing is affected, and if so, could this be due to the increase in Tra2b following \textit{UBA1} level increase? Do increased levels of the \textit{SMN} nuclear import protein Kpnb1 mean that the localisation of \textit{SMN} is altered in AAV9-%textit{UBA1} treated tissue? Do increases in the \textit{SMN} binding component of \textit{SNRNPs} Snrp3 mean that an improvement of snRNP assembly defects in SMA mice is seen? Whilst such intriguing outstanding questions still remain, together the data in this chapter provides novel insights in to molecular links between \textit{UBA1} and \textit{SMN}-dependent SMA and suggest that AAV9-%textit{UBA1} gene therapy is an exciting novel therapeutic approach for SMA.
Chapter 7. General discussion

7.1 Overview of results

In this thesis I have examined the role of the UPS in a range of SMA models and explored targeting of the UPS to develop novel therapeutic strategies in SMA mice. In particular I have addressed the following research aims:

1. Are UPS alterations a conserved molecular feature of different commonly used models of SMA?

In Chapter 3, I have shown that UPS alterations that have previously been described in the neuromuscular system of severe mouse models, in particular reduced levels of the E1 enzyme Uba1, are a feature in all major organ systems of Taiwanese SMA mice. These UPS alterations are also present in other SMA models including intermediate Smn2B/− mice, zebrafish and human patient derived iPSC motor neurons.

2. Do other β-catenin inhibitors, aside from quercetin, lead to improvements in SMA mice?

In Chapter 4, I have examined the effects of β-catenin inhibitors on their phenotype of SMA mice. In contrast to previous beneficial results with flavonoid compound quercetin, aspirin, indomethacin and iCRT-14 all failed to improve the weights, survival and motor performance of SMA mice. Likely off-target adverse effects mean that quercetin remains the most tolerable β-catenin inhibitor in SMA mice to date.

3. Does pharmacological inhibition of Uch1 improve the SMA mouse phenotype?

In Chapter 5, I have shown that pharmacological inhibition of the DUB Uch1 is not a viable therapeutic strategy in SMA mice failing to improve weights, motor performance, survival and neuromuscular pathology. Mimicking the SMA molecular phenotype in vitro suggested that increased Uch1 levels in SMA likely represent a compensatory response to reduced upstream Uba1 activity.
4. Is using gene therapy to increase UBA1 levels in SMA mice an effective therapeutic strategy?

In Chapter 6, I have shown that AAV9-UBA1 gene therapy is able to robustly increase UBA1 levels in SMA mice in vivo and leads to improvements of the weight, motor performance and NMJ pathology of SMA mice. Western blot and proteomic analysis also revealed that AAV9-UBA1 gene therapy is able to correct downstream UPS perturbations found in SMA as well as increasing SMN levels. Together this data suggests that UBA1 gene therapy is potentially an exciting novel therapeutic approach for SMA.

7.2 The UPS and SMA: therapies and future directions

The appropriate UPS control of protein degradation is important for maintaining normal cellular function. Disruption to UPS activity can lead to the abnormally accelerated breakdown of required substrates and/or the inappropriate stabilisation and accumulation of proteins. Understandably, therefore, aberrations in the UPS have long since known to be contribute to the pathogenesis of many diseases including neuromuscular and neurodegenerative disorders (reviewed by (Ciechanover and Brundin, 2003)). Indeed, an exciting novel mechanistic link between SMN and maintenance of ubiquitin homeostasis has also recently been discovered (Wishart et al., 2014). Previous research has found that levels of SMN protein are primarily regulated by the UPS (Chang et al., 2004) and that pharmacological inhibition of the proteasome (Kwon et al., 2011a) and inhibition of UPS downstream targets (Wishart et al., 2014) can lead to phenotypic improvements in SMA mice. In this thesis I have therefore focused on assessing if different aspects of the UPS that are perturbed in SMA could act as potential drug targets to develop new therapeutic strategies. In particularly I have focused on the E1 enzyme Uba1, the DUB Uchl1 and the downstream target β-catenin. Of these targets, Uba1 seems to have the most potential as increasing UBA1 levels using gene therapy was able to correct downstream UPS perturbations, increase SMN levels and lead to improvements in the phenotype of SMA mice.
Considering the *UBA1* loss-of-function mutations that cause X-linked SMA (Ramser et al., 2008, Dlamini et al., 2013, Jedrzejowska et al., 2015), it would also be pertinent to examine the therapeutic potential of UBA1 gene therapy on this related disease. However, due to *Uba1*’s complex location in mice (presence on the X-chromosome and additional copy on the Y-chromosome (Mitchell et al., 1991)) coupled with the lethality of gene knock out (Kulkarni and Smith, 2008) mean that no current mouse models of X-linked SMA exist. Importantly, reductions in SMN and Gemin 3 have been reported in X-linked SMA patient lymphoblasts (Yaeiz, 2008), indicating that as well as sharing clinical phenotypic characteristics, X-linked SMA and autosomal dominant *SMN*-dependent SMA may share a common underlying mechanism.

As ubiquitination is not the only regulator of protein homoeostasis it would be interesting to explore whether other ubiquitin-like modifiers are also altered in SMA. Ubiquitin-like systems such as interferon-stimulated gene-15 (ISG15), neural precursor cell-expressed developmentally downregulated-8 (NEDD8) and small ubiquitin-related modifier (SUMO) also influence the functions of proteins and can mediate their degradation (reviewed by (Herrmann et al., 2007)). Crosstalk between the UPS and ubiquitin-like systems exist with UBA1 itself having been shown to mediate NEDD8 activation under stress conditions (Leidecker et al., 2012) and also function in autophagy involving the ubiquitin-like protein autophagy-related protein 8 (Atg8) (Chang et al., 2013).

The poorly understood non-degradative roles of UBA1 would also be interesting to explore further, particularly its role in mitochondrial function and actin cytoskeleton signalling highlighted in the proteomic screen of AAV9-UBA1 treated hearts. Functional mitochondrial defects have been reported in a cell model of SMA (Acsadi et al., 2009) although it is not known if UPS alterations are a contributing factor or indeed if similar pathology is found *in vivo*. The link between UBA1, Plastin-3 and the actin cytoskeleton is an interesting one, and perhaps places SMA ubiquitination defects as a contributing factor to actin axonal perturbations caused by low SMN levels (Rossoll et al., 2003).
As recently reviewed by (Groen and Gillingwater, 2015), UBA1 defects have also been reported in other neurodegenerative disorders. In an *in vitro* assay using brain and peripheral organ tissue from a mouse model of Huntington’s disease, inhibition of Uba1 led to increased levels of polyglutamine expanded mutant huntingtin protein. The lower Uba1 expression in the brain compared with peripheral organs was found to correlate with the preferential accumulation of mutant huntingtin proteins in brain tissue (Wade et al., 2014). There was also a decline of Uba1 in the brain nuclear fraction with age also matching the preferential subcellular accumulation of mutant huntingtin (Wade et al., 2014). The authors argue that this selective reduction of Uba1 in the brain could contribute to the selective accumulation of mutant huntingtin and the selective neurodegeneration seen in Huntington’s disease (Wade et al., 2014).

In the cerebral cortex of Alzheimer’s disease patient post-mortem samples an *in vitro* assay showed reduced activity of E1-E2 thioester bond formation (Lopez Salon et al., 2000). Further analysis revealed a dramatic drop of UBA1 protein levels in the cytoplasmic fraction compared to normal levels in the particulate fraction (Lopez Salon et al., 2000). This suggests a delocalisation of UBA1 in Alzheimer’s disease patient brains similar in manner to that identified in SMA mouse motor neurons (Wishart et al., 2014). Furthermore, in a *Drosophila* model of fronto-temporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), a genetic screen revealed that Uba1 acts as a genetic modifier of Tau V337M toxicity (Blard et al., 2007). Pesticides that have been associated with an increased risk of developing Parkinson’s disease following exposure have been linked to dopaminergic cell damage as a result of Uba1 inhibition in fly and rodent models (Martin et al., 2014, Chou et al., 2008, Viquez et al., 2012). These studies suggest that environmental agents could also be affecting neurodegeneration by altering Uba1 dependent protein ubiquitination. Lastly, the P525L mutant FUS protein which causes severe juvenile ALS has increased UBA1 binding compared to the wildtype FUS protein (Wang et al., 2015). FUS- and TDP43-positive spinal neuron inclusions in ALS patients are known to co-localise with ubiquitin (Deng et al., 2010). One may therefore speculate that, as with SMA, altered UBA1 localisation maybe a feature of ALS motor...
neurons. Together these reports suggest that the work described in this thesis may potentially be applicable to other neurodegenerative conditions.

It is important to stress, however, that as reviewed in Chapter 1, the UPS is not the only molecular system perturbed in SMA. Many other SMN-dependent and SMN-independent strategies are established and continuing to emerge as therapeutic avenues. Encouraging reports from ongoing clinical trials of ASOs, and more recently SMN-gene therapy, are important developments for the SMA field. Recent advances in genome editing technology, such as improvements in the CRISPR/Cas system, may also soon make SMN2 to SMN conversion possible at the DNA level. Overwhelming evidence of systemic defects reported in SMA is also likely to take the sole focus of SMA therapy development away from the motor neuron. How UPS alterations in SMA intertwine with other known SMA cellular pathologies to eventually result in the clinical phenotype caused by low SMN levels remains to be fully investigated. Ultimately, it may be that correcting UPS perturbations in SMA can be used to therapeutic benefit either as an independent strategy or in combination with other strategies to maximise their effectiveness.

7.3 Conclusion

In this thesis I have examined the role UPS dysfunction in a range of SMA models and explored targeting different components of the UPS to develop novel therapeutic strategies in SMA mice. Together, this work has contributed to further understanding molecular alterations underlying SMA pathogenesis and to the development of novel SMN-independent therapies.
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Appendices

Appendix 1. AAV9-GFP delivery did not increase Uba1 levels in any organ and all time points studied

Analysis of Uba1 protein in CD1 wildtype mice following an intravenous injection of AAV9-GFP at P1. Western blot of GFP protein levels in the brain, heart, liver, lung, kidney, muscle and spinal cord (cervical, thoracic and lumbar levels) of wildtype CD1 mice culled at P3, P4, P6, P7, P8, P30 and P60 following injection of AAV9-GFP via the facial vein at P1 (+) and uninjected controls (-). Dotted lines represent image processing to align lane well order.
Appendix 2. AAV9-GFP delivery did not alter the body weight or motor performance of CD1 wild type mice

Phenotype analysis of CD1 wildtype mice following an intravenous injection of AAV9-GFP at P1. **A-B.** Graph showing the weights (**A**) and righting time (**B**) of CD1 wildtype mice overtime following an intravenous injection of AAV9-GFP at P1. Data shown at mean ± SEM. N=8 at P1-3, N=7 at P4, N=6 at P5, N=5 at P6, N=4 at P7, N=3 at P8, N=2 at P30 and N=1 at P60 for each treatment group. ns = not significant; unpaired, two-tailed Student’s t-test.
Appendix 3. Intravenous injection of AAV-UBA1 is safe in control mice in the long-term

No overt adverse effects noted in control mice treated mice which received an intraperitoneal injection of AAV9-UBA1 at P1. A. Weights of P30 (N=11) and P60 (N=13) uninjected control littermates and P30 (N=11) and P60 (N=8) control littermates that received an intravenous injection of AAV9-UBA1 via the facial vein at P1. Data shown at mean ± SEM. ns = not significant; unpaired, two-tailed Student's t-test. B. Representative photographs of P30 control littermates, uninjected (top panel) and AAV9-UBA1 injected (bottom panel). Scale bar = 2cm.
Appendix 4. Haematology analysis of blood from P30 uninjected control littermate mice and P30 control littermates which had received an intravenous injection of AAV9-UBA1 at P1

<table>
<thead>
<tr>
<th>Haematology</th>
<th>Units</th>
<th>Uninjected control</th>
<th>AAV9-UBA1</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cells</td>
<td>x10^9/l</td>
<td>1.9 ± 0.4</td>
<td>2.0 ± 0.2</td>
<td>0.876</td>
</tr>
<tr>
<td>Neutrophils (segmented)</td>
<td>x10^9/l</td>
<td>0.186 ± 0.049</td>
<td>0.145 ± 0.016</td>
<td>0.476</td>
</tr>
<tr>
<td>Neutrophils (segmented)</td>
<td>%</td>
<td>9.3 ± 0.9</td>
<td>7.3 ± 0.9</td>
<td>0.184</td>
</tr>
<tr>
<td>Neutrophils (non-segmented)</td>
<td>x10^9/l</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>Neutrophils (non-segmented)</td>
<td>%</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>x10^9/l</td>
<td>1.743 ± 0.328</td>
<td>1.85 ± 0.150</td>
<td>0.786</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>%</td>
<td>90.3 ± 0.6</td>
<td>92.3 ± 0.7</td>
<td>0.101</td>
</tr>
<tr>
<td>Monocytes</td>
<td>x10^9/l</td>
<td>0.004 ± 0.004</td>
<td>0.333 ± 0.333</td>
<td>0.379</td>
</tr>
<tr>
<td>Monocytes</td>
<td>%</td>
<td>0.3 ± 0.3</td>
<td>0</td>
<td>0.373</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>x10^9/l</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>%</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>Basophils</td>
<td>x10^9/l</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>Basophils</td>
<td>%</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>x10^12/l</td>
<td>7.42 ± 0.42</td>
<td>6.98 ± 0.12</td>
<td>0.365</td>
</tr>
<tr>
<td>Packed cell volume</td>
<td>l/l</td>
<td>0.379 ± 0.022</td>
<td>0.361 ± 0.010</td>
<td>0.512</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>g/dl</td>
<td>12.4 ± 0.7</td>
<td>12.0 ± 0.3</td>
<td>0.625</td>
</tr>
<tr>
<td>Mean corpuscular volume</td>
<td>fl</td>
<td>51.0 ± 0.0</td>
<td>51.7 ± 0.9</td>
<td>0.492</td>
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<tr>
<td>Mean corpuscular haemoglobin</td>
<td>%</td>
<td>32.8 ± 0.1</td>
<td>33.3 ± 0.4</td>
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<tr>
<td>Red cell distribution width</td>
<td>%</td>
<td>14.4 ± 0.4</td>
<td>13.8 ± 0.1</td>
<td>0.214</td>
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</table>

Data shown at mean ± SEM. N=3 for each treatment group. Groups compared using an unpaired, two-tailed Student’s t-test
Appendix 5. Blood serum biochemistry analysis of blood from P30 uninjected control littermate mice and P30 control littermates which had received an intravenous injection of AAV9-UBA1 at P1

<table>
<thead>
<tr>
<th>Serum Biochemistry</th>
<th>Units</th>
<th>Uninjected control</th>
<th>AAV9-UBA1</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Albumin</td>
<td>g/l</td>
<td>26.2 ± 1.5</td>
<td>25.4 ± 0.9</td>
<td>0.651</td>
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<tr>
<td>Alanine aminotransferase</td>
<td>IU/L</td>
<td>48 ± 3</td>
<td>52 ± 2</td>
<td>0.436</td>
</tr>
<tr>
<td>Bile Acids</td>
<td>µmol/l</td>
<td>1.1 ± 0.3</td>
<td>0.8 ± 0.4</td>
<td>0.521</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>µmol/l</td>
<td>1.3 ± 0.1</td>
<td>2.2 ± 1.9</td>
<td>0.673</td>
</tr>
<tr>
<td>Chloride</td>
<td>mmol/l</td>
<td>102 ± 6</td>
<td>94 ± 4</td>
<td>0.343</td>
</tr>
<tr>
<td>Creatine</td>
<td>µmol/l</td>
<td>42 ± 1</td>
<td>39 ± 1</td>
<td>0.100</td>
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<tr>
<td>Globulin</td>
<td>g/l</td>
<td>15.8 ± 1.4</td>
<td>14.9 ± 1.0</td>
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</tr>
<tr>
<td>Inorganic phosphate</td>
<td>mmol/l</td>
<td>3.41 ± 0.01</td>
<td>3.64 ± 0.43</td>
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<tr>
<td>Potassium</td>
<td>mmol/l</td>
<td>7.4 ± 1.1</td>
<td>8.2 ± 0.8</td>
<td>0.592</td>
</tr>
<tr>
<td>Protein</td>
<td>g/l</td>
<td>42.0 ± 2.9</td>
<td>40.3 ± 1.8</td>
<td>0.650</td>
</tr>
<tr>
<td>Urea</td>
<td>mmol/l</td>
<td>8.0 ± 0.8</td>
<td>7.3 ± 0.4</td>
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</tbody>
</table>

Data shown at mean ± SEM. N=3 for each treatment group. Groups compared using an unpaired, two-tailed Student’s t-test
### Proteins with increased >20% expression in AAV9-UBA1 treated P7 mouse hearts compared to uninjected controls

<table>
<thead>
<tr>
<th>Accession ID</th>
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<th>Protein name</th>
<th>Unique peptides</th>
<th>Score</th>
<th>Anova (P)</th>
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<td>B9EHN0</td>
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<td>Corrin 1a</td>
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<td>S100A9</td>
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<td>Q7FTF1</td>
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<td>Catepsin C</td>
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<td>AM6UV1</td>
<td>Ybar1</td>
<td>Alpha-globin</td>
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<td>331</td>
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<td>Q05734</td>
<td>Sepsin3m</td>
<td>Serine protease inhibitor A3M</td>
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<td>Ybb1</td>
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<td>O84477</td>
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<td>Fibrinogen gamma chain</td>
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<td>Cpi</td>
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<td>Apoc4</td>
<td>Actin-related protein 2/3 complex subunit 4</td>
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<tr>
<td>Q6PEO6</td>
<td>Atp6v10</td>
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<tr>
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<td>Alanine--RNA ligase</td>
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<td>Fibrinogen, B beta polypeptide, isoform CRA_a</td>
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<td>Myosin-9</td>
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<td>Filamin-C</td>
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### Appendix 7. Proteins with decreased >1.2 fold expression only in AAV9-UBA1 P7 control mouse hearts compared to uninjected controls

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<th>Accession ID</th>
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<th>Protein name</th>
<th>Unique peptides</th>
<th>Score</th>
<th>Anova (P)</th>
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<tr>
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<td>Atylkine kinase ADCK3, mitochondrial</td>
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<tr>
<td>Z3UQ59</td>
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<td>Myosin-2</td>
<td>23</td>
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<tr>
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<td>Rypine peptides 2</td>
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<td>Myosin light chain kinase 3</td>
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<td>Mdy6c3</td>
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<td>Protein S100</td>
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<td>A2AFW6</td>
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<td>Mitochondrial carnitine hormone 2</td>
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## Appendix 8. Proteins with increased >1.2 fold expression in both AAV9-UBA1 and AAV9-GFP P7 control mouse hearts compared to uninjected controls

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<td>Q3V1A8</td>
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<td>P01125</td>
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<td>ATPase mitochondrial</td>
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Proteins with increased >1.2 fold expression only in AAV9-GFP P7 control mouse hearts compared to uninjected controls

<table>
<thead>
<tr>
<th>Accession ID</th>
<th>Gene name</th>
<th>Protein name</th>
<th>Unique peptides</th>
<th>Score</th>
<th>Anova (P)</th>
<th>Fold change: AAV9-GFP/uninjected</th>
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<tbody>
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<td>C5H0E8</td>
<td>Gm9392</td>
<td>Rap1A-retro1</td>
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<td>Q99LC3</td>
<td>Ndufa10</td>
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<td>Q1TIE8</td>
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<td>H3BKA1</td>
<td>Acaa1a</td>
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<td>254</td>
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Appendix 10. Proteins with decreased >-1.2 fold expression only in AAV9-UBA1 P7 control mouse hearts compared to uninjected controls

<table>
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<tr>
<th>Accession ID</th>
<th>Gene name</th>
<th>Protein name</th>
<th>Unique peptides</th>
<th>Score</th>
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<th>Fold change: AAV9-GFP/uninjected</th>
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<td>B2F0X9</td>
<td>Mya7</td>
<td>Myosin, heavy polypeptide 7, cardiac muscle, beta</td>
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Appendix 11. Top canonical pathways identified by Ingenuity Pathway Analysis (IPA) proteomic analysis of proteins with a >20% change in both AAV9-UBA1 and AAV9-GFP P7 control hearts

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<th>Name</th>
<th>P-value</th>
<th>Overlap</th>
<th>Individual proteins</th>
</tr>
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<tbody>
<tr>
<td>Acute phase response signaling</td>
<td>2.80E-05</td>
<td>3.0% (5/169)</td>
<td>HPX, HP, APOA1, FGA, A2M</td>
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<tr>
<td>Polyamine regulation in colon cancer</td>
<td>9.84E-04</td>
<td>9.1% (2/22)</td>
<td>PSME1, PSME2</td>
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<tr>
<td>Glycolysis I</td>
<td>1.27E-03</td>
<td>8.0% (2/25)</td>
<td>ALDOB, PFKM</td>
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<tr>
<td>LXR/RXR activation</td>
<td>2.14E-03</td>
<td>2.5% (3/121)</td>
<td>HPX, APOA1, FGA</td>
</tr>
<tr>
<td>Interferon signaling</td>
<td>2.35E-03</td>
<td>5.9% (2/34)</td>
<td>IFITM3, STAT1</td>
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</table>
Appendix 12. Top canonical pathways identified by Ingenuity Pathway Analysis (IPA) proteomic analysis of proteins with a >20% change in only AAV9-GFP P7 control hearts

<table>
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<th>Name</th>
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<th>Individual proteins</th>
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<tr>
<td>TCA cycle II (eukaryotic)</td>
<td>5.17E-07</td>
<td>13.0% (3/23)</td>
<td>SUCLA2, DLD, FH</td>
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<td>Valine degradation I</td>
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<td>2-ketoglutarate dehydrogenase complex</td>
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<td>25.0% (1/4)</td>
<td>DLD</td>
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<tr>
<td>Branched chain keto acid dehydrogenase complex</td>
<td>2.95E-03</td>
<td>25.0% (1/4)</td>
<td>DLD</td>
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Appendix 13. No change in UBA1 or SMN protein levels in P7 SMA and control littermates following intravenous injection of AAV9-GFP via the facial vein at P1

Analysis of SMN protein and UBA1 proteins in P7 SMA and control littermates following intravenous injection of AAV9-GFP via the facial vein at P1. A. Western blots showing GFP (top panel) and UBA1 (upper middle panel) and SMN (lower middle panel) protein levels in the heart of P7 uninjected control and SMA mice (-) and P7 control and SMA mice which had received an intravenous injection of AAV9-UBA1 at P1 (+). Bottom panel shows α-tubulin loading. B-D. Bar charts showing the quantification of GFP (B), UBA1 (C) and SMN (D) protein levels in the heart of P7 uninjected control and SMA mice and P7 control and SMA mice which had received an intravenous injection of AAV9-GFP at P1. Protein levels are expressed as a percentage of uninjected control levels and are shown as mean ± SEM. N=3 for each treatment group. ***P<0.005, ****P<0.001, ns = not significant; unpaired, two-tailed Student’s t-test.
Appendix 14. Publications


Powis RA, Gillingwater TH. 2015. Selective loss of alpha motor neurons with sparing of gamma motor neurons and spinal cholinergic neurons in a mouse model of spinal muscular atrophy. Accepted for publication at the Journal of Anatomy.