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THE UNIVERSITY  
*of* EDINBURGH

**Investigating modifiers that can regulate  
selective vulnerability in mouse models  
of spinal muscular atrophy**

Victoria Woschitz

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A thesis submitted for the degree of PhD

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## Declaration

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I declare that the work presented in this thesis is entirely my own, unless otherwise stated in the text, and has not been submitted for any other degree or professional qualification.

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Victoria Woschitz

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## Abstract

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Spinal muscular atrophy (SMA) is a childhood form of motor neuron disease. It is characterised by the loss of lower motor neurons and muscle weakening and atrophy of associated muscles. Motor neurons (MNs) are the primary pathological target of SMA, but it has long been shown that breakdown of neuromuscular junctions (NMJs) is also an early pathological target of the disease. However, not all MNs are equally vulnerable, with some being lost very early in the course of the disease, whilst other subpopulations are consistently spared until late stages of the disease. This selective vulnerability of MNs is a well-known pathological hallmark of SMA, however the underlying molecular mechanisms of it are yet unknown. The work contained within this thesis aims to investigate the basis of selective vulnerability, which will give critical insight into the factors which drive motor neuron pathology and help identify novel neuroprotective strategies.

Selective vulnerability of MNs and a wide range of muscles has been reported in SMA patients and in commonly utilised mouse models of SMA. Despite the many publications in each mouse model of SMA, there has not yet been a comparison of NMJ pathology across different mouse models of SMA. I therefore performed an extensive analysis of NMJ pathology in 20 muscles from the *Smn*<sup>2B/-</sup> mouse model of SMA and subsequently compared pattern of selective vulnerability between other mouse models of SMA. This revealed a more profound motor neuron cell body loss in thoracic and upper lumbar spinal cord segments and higher levels of NMJ loss in muscles innervated by nerves from these spinal cord segments in the *Smn*<sup>2B/-</sup> mouse model. Comparison of selective vulnerability patterns between the *Smn*<sup>2B/-</sup> mouse model and other mouse models of SMA, showed that all models had a distinct pattern of selective vulnerability, with the *Smn*<sup>2B/-</sup> showing most similarity to patients. Patterns of vulnerability could not be attributed to developmental subtype or muscle fibre type in any of the mouse models.

To gain insight into the molecular mechanisms regulating selective vulnerability, I performed cross-model transcriptional analysis. Here, my goal

was to analyse published RNA-seq data sets to look for novel modifiers. I obtained RNA-seq data sets from laser capture microdissection (LCM) motor neurons from both SMN $\Delta$ 7 and *Smn*<sup>2B/-</sup> mice, from a range of resistant and vulnerable populations. I reanalysed and compared these data sets and identified a number of exciting potential modifiers. In particular, pituitary adenylate cyclase-activating polypeptide (PACAP), seemed to be an exciting potential neuroprotective modifier.

PACAP has been shown to be neuroprotective in an array of neurodegenerative diseases but has not yet been investigated in models of SMA. Here, I investigated whether increased PACAP levels could ameliorate neuronal pathology in a mouse model of SMA. Firstly, PACAP was administered IP to the *Smn*<sup>2B/-</sup> mouse model, showing no significant effect on survival, motor performance and selective vulnerability. Therefore, subsequently PACAP was administered via AAV9 delivery in the *Smn*<sup>2B/-</sup> mouse model, showing no significant effects on the neuropathology in the *Smn*<sup>2B/-</sup> mouse model.

Finally, since I have identified significant divergence in relative vulnerability in the neurons of the facial motor nucleus, I aimed to perform high spatial resolution transcriptional profiling of motor neurons in the SMN $\Delta$ 7 mouse model with the ultimate goal of identifying transcriptional signatures of differential vulnerability. I therefore optimised and applied a novel method to perform RNA sequencing on single MNs isolated from the facial nucleus of the brainstem by LCM. A total of 1050 single MNs have been isolated from the facial nucleus from individual P2, P5 and P9 SMN $\Delta$ 7 mice and respective controls. cDNA libraries were prepared and cDNA fragmented, uniquely barcoded and sent for initial quality control RNA-seq analysis. The analysis revealed promising quality control parameters of the sequenced samples and thereby confirmed a successful optimisation of this novel sequencing technique.

Overall, these results provide an important insight into selective vulnerability patterns of different SMA mouse models and identified novel mechanisms and

transcripts that have the potential to contribute to the protection of motor neurons in SMA mouse models.

## Lay Abstract

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Motor neurons are long nerve cells that connect the brain and spinal cord with the muscles of our bodies. These nerve cells are responsible for sending commands from the brain to the spinal cord and then to the muscles, to allow us to move, breathe, swallow and talk. If these nerve cells become damaged by disease, these commands get interrupted and the communication with the muscles become impaired and eventually breaks down. This is what happens in motor neuron disease like spinal muscular atrophy (SMA) – the motor neurons break down, or degenerate, and lose their communication and their point of connection to the muscle, which is called the neuromuscular junction (NMJ). This means that because patient's muscles lose their connection to the spinal cord, muscles are no longer able to sufficiently contract and perform movement. This in turn leads to weakness and wasting of the muscles and eventually to muscle paralysis in patients. We know that in SMA patients, a lot of these motor neurons degenerate leaving many muscles weak or paralysed. However, interestingly not all motor neurons and their connected muscles are equally affected by the disease. Some motor neurons and associated muscles are lost early during the disease, while others stay relatively intact until late stages of the disease. This phenomenon is called 'selective vulnerability' of muscles. Selective vulnerability of muscles is present in all SMA patients and also in mouse models of SMA. Importantly, it is not yet determined why some muscles are more affected than others. This thesis aims to better understand which factors can influence this selective vulnerability in SMA. Factors that influence and alter selective vulnerability are interesting because targeting these factors could represent a treatment to prevent or slow the degeneration of motor neurons. Here, I investigated the pattern of muscles that show selective vulnerability in four different mouse models of SMA and showed that each mouse model shows a different pattern of selective vulnerability. These findings can help us select the best experimental approach to research specific aspects of the disease. Further, I analysed data from different SMA mouse models to identify factors that could potentially influence selective vulnerability. I found that there are many potential factors that could be tested as a treatment

to slow or prevent motor neuron breakdown. One specific factor, called PACAP, looked like the most promising candidate to test in a mouse model of SMA. I administered PACAP treatment to mice with two different methods and investigated the effect of the treatment on the general health of the animals and on their muscle performance. Lastly, I optimised an experimental method that allows us to collect individual motor neurons from the brain of animals from a mouse model of SMA. I collected and prepared 1050 individual motor neurons to be sent for complex collection of genetic data. This method can help us to further identify a refined list of potential factors that can influence selective vulnerability in SMA, helping us to eventually discover new treatments for SMA patients.

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## List of Abbreviations

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|                 |                                                                 |
|-----------------|-----------------------------------------------------------------|
| μm              | Micrometre                                                      |
| μM              | Micromolar                                                      |
| μL              | Microliter                                                      |
| AAL             | Adductor auris longus                                           |
| AAV9            | Adeno-associated virus 9                                        |
| AChR            | Acetyl choline receptor                                         |
| ADBE            | Activity-dependent bulk endocytosis                             |
| ALS             | Amyotrophic lateral sclerosis                                   |
| AS              | Auricularis superior                                            |
| ASO             | Antisense oligonucleotide                                       |
| BC              | Biceps brachii                                                  |
| BSA             | Bovine Serum Albumin                                            |
| BTX             | Bungarotoxin                                                    |
| CB              | Cajal body                                                      |
| cDNA            | Complementary DNA                                               |
| ChAT            | Choline acetyltransferase                                       |
| CN III          | Cranial nerve nucleus III                                       |
| CN IV           | Cranial nerve nucleus IV                                        |
| CN VII          | Cranial nerve nucleus VII                                       |
| CN XII          | Cranial nerve nucleus XII                                       |
| CNS             | Central nervous system                                          |
| CO <sub>2</sub> | Carbon dioxide                                                  |
| DAPI            | 4',6-Diamidine-2'-phenylindole dihydrochloride                  |
| DAVID           | Database for annotation, visualisation and integrated discovery |
| DE              | Deltoid                                                         |
| DeSyn           | Delayed synapsing                                               |
| DEG             | Differentially expressed gene                                   |
| DNA             | Deoxyribonucleic Acid                                           |
| DP              | Digastric posterior                                             |
| dsDNA           | Double stranded DNA                                             |

|        |                               |
|--------|-------------------------------|
| EDL    | Extensor digitorum longus     |
| EMA    | European Medicines Agency     |
| EO     | External oblique              |
| ES     | Enrichment score              |
| FaSyn  | Fast synapsing                |
| FCR    | Flexor carpi radialis         |
| FDA    | Food and Drug Administration  |
| FDB    | Flexor digitorum brevis       |
| g      | Grams                         |
| GABA   | Gamma-aminobutyric acid       |
| GAP-43 | Growth-associated protein 43  |
| GEO    | Gene expression omnibus       |
| GlyR   | Glycine receptor              |
| GO     | Gene ontology                 |
| GR     | Gracilis                      |
| GS     | Gastrocnemius                 |
| IC     | Intercostals                  |
| ICV    | Intracerebroventricular       |
| IP     | Intraperitoneal               |
| LALc   | Levator auris longus caudal   |
| LALr   | Levator auris longus rostral  |
| LCM    | Laser capture microdissection |
| LU     | Lumbricals                    |
| M      | Molar                         |
| mg     | Milligram                     |
| mL     | Millilitre                    |
| mm     | Millimetre                    |
| mM     | Millimolar                    |
| MA     | Masseter                      |
| MAR    | Missing at random             |
| MN     | Motor neuron                  |
| MND    | Motor neuron disease          |
| mRBP   | mRNA binding proteins         |
| MRI    | Magnetic resonance imaging    |

|                |                                                    |
|----------------|----------------------------------------------------|
| mRNA           | Messenger ribonucleic acid                         |
| mRNP           | Messenger ribonucleoproteins                       |
| NF             | Neurofilament                                      |
| NFA            | Neurofilament accumulation                         |
| ng             | Nanogram                                           |
| nM             | Nanomolar                                          |
| NMJ            | Neuromuscular junction                             |
| OCT            | Optimal cutting temperature compound               |
| P              | Postnatal day (e.g. postnatal day 18 = P18)        |
| PACAP          | Pituitary adenylate cyclase-activating polypeptide |
| PBS            | Phosphate-buffered saline                          |
| PCA            | Principal component analysis                       |
| PCR            | Polymerase Chain Reaction                          |
| PEN            | Polyethylene naphthalate                           |
| PFA            | Paraformaldehyde                                   |
| PLS            | Primary lateral sclerosis                          |
| pM             | Picomolar                                          |
| PMM            | Predictive mean matching                           |
| PNS            | Peripheral nervous system                          |
| QC             | Quadriceps                                         |
| RA             | Rectus abdominis                                   |
| RNA            | Ribonucleic acid                                   |
| ROCK           | Rho-associated kinase pathway                      |
| rpm            | Rounds per minute                                  |
| rRNA           | Ribosomal ribonucleic acid                         |
| SCM            | Sternocleidomastoid                                |
| SEM            | Standard error of the mean                         |
| SH             | Sternohyoid                                        |
| SMA            | Spinal muscular atrophy                            |
| <i>SMN/Smn</i> | Survival motor neuron gene (HUMAN/murine)          |
| SMN/Smn        | Survival motor neuron protein (HUMAN/murine)       |
| <i>SMN1</i>    | Survival motor neuron 1                            |
| <i>SMN2</i>    | Survival motor neuron 2                            |
| snRNA          | Small nuclear RNA                                  |

|        |                                  |
|--------|----------------------------------|
| snoRNA | Small nucleolar RNA              |
| snRNP  | Small nuclear ribonucleoproteins |
| SO     | Soleus                           |
| SV2    | Synaptic vesicle protein 2       |
| TA     | Tibialis anterior                |
| TC     | Triceps brachii                  |
| TMR    | Tetramethylrhodamine             |
| TTLB   | Tail tip lysis buffer            |
| TTR    | Time to right test               |
| TS     | Triangularis sterni              |
| tSC    | Thoracic spinal cord             |
| TTT    | Time to turn around test         |
| TVA    | Transversus abdominis            |
| U      | Units                            |
| VG     | Viral Genomes                    |
| WT     | Wild type                        |

# Chapter 1

---

## General Introduction

Motor neuron disease (MND) is an umbrella term encompassing diseases that affect upper and lower motor neurons. The term MND is sometimes used interchangeably with amyotrophic lateral sclerosis (ALS), even though more diseases than ALS fall under this terminology. Aside from ALS, MND also includes the diseases primary lateral sclerosis (PLS) and spinal muscular atrophy (SMA). Even though the aetiologies of these diseases are different, they all have one thing in common: their degeneration and loss of motor neurons. There are upper and lower motor neurons that carry signals from the brain to the muscle, enabling movement of the respective muscle. Upper motor neurons carry signals from the motor cortex to the brainstem or the ventral horn of the spinal cord. Lower motor neurons are the connections between the central nervous system (CNS) and the muscles, carrying signals from the upper motor neurons to the muscles, thereby enabling muscle contractions. While in ALS both upper and lower motor neurons are affected, in PLS only upper motor neurons are affected, and in SMA only lower motor neurons are affected (Talbot, 2002).

Despite sharing motor neuron loss as their major hallmark of the disease, the aetiology of all MNDs is distinct. ALS is caused by mutations in a variety of genes, including *C9ORF72*, *FUS*, *SOD1*, and *TARDBP* (Feldman et al., 2022). It can be of sporadic origin, and in a minority of cases (10-15%) familial, with a mean disease onset of 62 years and a mean life expectancy of 2-5 years after diagnosis (as reviewed by Feldman et al., 2022; Hulisz, 2018). Patients experience motor neuron loss and this dysfunction leads to progressive weakness of voluntary skeletal muscles involved in limb movement, swallowing, speaking, and respiratory function (Feldman et al., 2022). PLS is a rare form of MND, making up less than 3% of all MND cases (Turner and Talbot, 2020). PLS shares the pathological signature of ALS, and the diagnosis

is therefore purely clinical, based on the characteristic features of the disease. These features include a slowly progressive spasticity that begins in the lower limbs, or in rare cases with spastic dysarthria, and muscle stiffness and weakness (as reviewed by Turner and Talbot, 2020). PLS has an adult disease onset, typically presenting around the age of 50 years, and is in many cases not fatal but slowly progressing with a prognosis of at least a decade or often significantly longer (Turner and Talbot, 2020). In contrast to ALS and PLS that can have a sporadic origin, SMA is an inherited autosomal recessive disorder, whose genetic cause is well documented (Lefebvre et al., 1995). The in-depth knowledge about the genetic makeup of this disease allowed the development of several pre-clinical, including murine, models to study the mechanisms of this disease, and further led to the development of three approved therapies for SMA patients.

### **1.1. Spinal muscular atrophy – an overview**

The childhood motor neuron disease, spinal muscular atrophy (SMA), is an autosomal recessive disorder that is characterised by the loss of lower alpha motor neurons from the ventral horn of the spinal cord. It presents as progressive muscle weakness, atrophy and ultimately muscle paralysis (Lunn and Wang, 2008). SMA is the leading genetic cause of infant mortality with incidence traditionally considered to be 1 in 6,000-10,000 live births (Lunn and Wang, 2008). More recent data suggests that SMA has an incidence of 1 in 3,900-16,000 live births in Europe alone (Verhaart et al., 2017).

#### **1.1.1. The genetics of SMA**

SMA is caused by homozygous deletions and mutations in the survival motor neuron 1 (*SMN1*) gene that is located on the telomeric part of chromosome 5q13. Mutations in this gene lead to the disruption of the production of the ubiquitously expressed survival motor neuron (SMN) protein, which is essential for the survival of motor neurons. In 95% of SMA patients a deletion of the *SMN1* gene is present, causing the disease (Lefebvre et al., 1995). On the centromeric part of an inverted duplication of chromosome 5q13, lies the survival motor neuron 2 (*SMN2*) gene (Rochette et al., 2001). The *SMN1* and *SMN2* genes are almost identical, both containing 9 exons, 8 introns and

showing differences at only 5 bases. The only functional difference between the genes is a C to T translation within exon 7 of the *SMN2* gene (Figure 1.1). This critical substitution difference disrupts the exon splicing enhancer site, leading to an alternative splicing and skipping of exon 7 in the *SMN2* gene (Lorson et al., 1999; Singh et al., 2017; Singh and Singh, 2018). The alternative splicing alters the amount of exon 7 that is incorporated in the final SMN transcript, with most transcripts from *SMN2* lacking exon 7. This results in the gene only producing around 10% of functioning full-length SMN protein, leaving most of the produced protein to be an unstable, truncated, rapidly degrading version of the protein, termed SMN $\Delta$ 7 (Lorson et al., 1999; Singh and Singh, 2018). As a result, there is a reduced amount of functional, full-length SMN protein in SMA, which is not sufficient to support physiological function of the neuromuscular system (Figure 1.1). This leads to degeneration of motor neurons and associated muscles, thereby leading to muscle weakness and paralysis and ultimately, in severe cases, to death under the age of 2 years (Zerres and Rudnik-Schöneborn, 1995).

While a complete lack of full-length Smn, following a loss of *Smn*, was proven to be embryonically lethal in mice (Schränk et al., 1997), the small amounts of full-length SMN produced by *SMN2* can be responsible for sustaining life. It has been shown that the number of *SMN2* copies in the human genome is variable, and that it inversely correlates with disease severity (Feldkötter et al., 2002; McAndrew et al., 1997; Wadman et al., 2017; Wirth et al., 2006). This means that in individuals with deletion of *SMN1*, the *SMN2* copy number, and the small amount of functional full-length SMN produced by each *SMN2* copy, are determinants for the SMA phenotype severity. Generally, higher numbers of *SMN2* copies correlate with a milder disease phenotype (Feldkötter et al., 2002; Wadman et al., 2017; Wirth et al., 2006).

*SMN2* has been described as a phenotypic disease modifier of SMA, making splicing modifications of *SMN2* a well-utilised mechanism of action for therapeutics in SMA, as higher SMN levels can extend survival. This has led to the approval of Nusinersen (Spinraza) as a treatment of SMA (see section 1.1.3. for detailed description) (Finkel et al., 2017; Mercuri et al., 2018).

However, while variations in *SMN2* copy number are the leading cause for disease severity, there are other factors that act as phenotypic disease modifiers, including plastin 3 and neurocalcin delta (Oprea et al., 2008; Riessland et al., 2017; Wadman et al., 2020). Plastin 3, an actin-binding protein that is of importance in axonogenesis, was shown to rescue axon length and outgrowth defects associated with SMA when overexpressed in pre-clinical models (Oprea et al., 2008). Further, neurocalcin delta, which when knocked down ameliorated SMA-associated pathological defects, has been identified as other phenotypic modifier of SMA (Riessland et al., 2017). Consequently, although *SMN2* is a primary modifier of severity, other genetic factors can have an impact on disease manifestation.

Full-length functioning SMN protein contains 294 amino acids and has a molecular weight of 38kDa. It is a ubiquitously expressed protein, therefore being expressed in all types of tissues. It remains unknown why reduced levels of SMN lead to a specific motor neuron disease phenotype in SMA, given that the CNS is not the only tissue where the protein is expressed. However, the levels of SMN present can vary greatly between cell types and tissues. It has been shown that Smn protein expression was highly influenced by age and tissue type in control mice (Groen et al., 2018). Further, higher levels of Smn were found in CNS, specifically spinal motor neurons in mice (Groen et al., 2018; Monani, 2005).

SMN is located in the cytoplasm and nucleus of cells (Gubitz et al., 2004). In the nucleus, SMN is enriched within bodies called “gems”, which stands for “gemini of cajal bodies” (Gubitz et al., 2004; Liu and Dreyfuss, 1996). Gems consist of SMN complex proteins and have a similar size and number to Cajal bodies (CBs) with which they are often associated (Liu and Dreyfuss, 1996). SMN also localises to Cajal bodies, which are more complex structures of the nucleus. CBs have an enrichment in factors that are involved in the transcription and processing of different types of nuclear RNAs, including small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs). They are essential for the biogenesis of the small nuclear ribonucleoproteins (snRNP) complex.

SMN protein is implicated in various cellular processes and pathways, but its most studied and well-understood role is in the assembly of snRNPs and pre-mRNA splicing (as reviewed by Burghes and Beattie, 2009; Chaytow et al., 2018; Monani, 2005). Pre-mRNA splicing is the process of removing non-coding introns from the pre-mRNA transcripts and re-joining the remaining coding exons to allow a progression of the translation process. Protein complexes called spliceosomes facilitate this splicing process. A large component of these spliceosomes are snRNPs, in the assembly of which SMN was shown to play a role via the SMN complex. The SMN complex is comprised of SMN, Gemins 2-8, unr interacting proteins (unrip), and ATP (Burghes and Beattie, 2009). It binds Sm proteins to snRNAs, and the whole SMN complex together with the snRNAs is transported into the nucleus (Burghes and Beattie, 2009; Chaytow et al., 2018; Monani, 2005). There, snRNPs are further matured in preparation for pre-mRNA splicing (Burghes and Beattie, 2009). In short, the role of SMN protein, that is associated with the SMN complex, is to facilitate the biogenesis of snRNPs from snRNAs in the cytoplasm, followed by subsequent snRNP trafficking to the nucleus for pre-mRNA splicing. An SMN-dependent deficiency in the formation of RNPs is seen in SMA, thereby best describing SMA as an RNP hypo-assembly disease (as reviewed by Price et al., 2018).

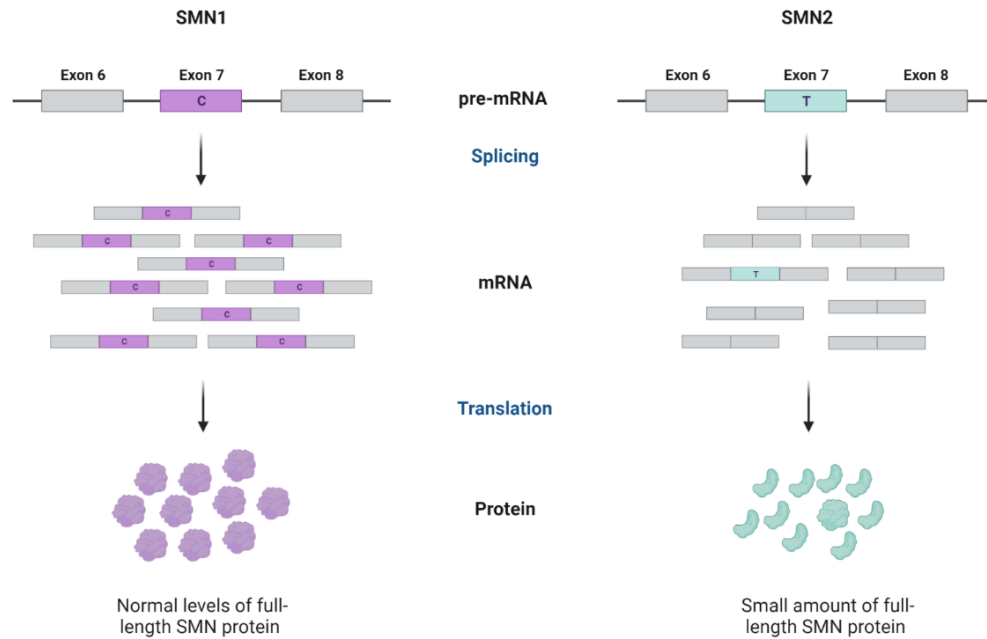
Although pre-mRNA splicing is the best characterised function of SMN, the protein has also been implicated in RNA trafficking, implicating that it mediates the axonal localisation of mRNA-protein complexes, which could in turn contribute to the axonal degeneration in SMA. SMN was shown to be present as granules in the growth cones of motor neurons in culture, and it was demonstrated that SMN was transported both in an anterograde and retrograde direction along the motor neuron axons (Zhang et al., 2003). It was first suggested that SMN has a role in  $\beta$ -actin mRNA trafficking, when it was revealed that SMN contributes to the localisation of  $\beta$ -actin mRNA to growth cones (Rossoll et al., 2003). It was further shown that a deficiency in SMN led to impairments in neurite outgrowth and the localisation of  $\beta$ -actin mRNA to growth cones (Rossoll et al., 2003). It has been suggested that SMN acts as molecular chaperone for the assembly of messenger ribonucleoproteins

(mRNPs) from mRNA and mRNA binding proteins (mRBPs), binding RBPs to mRNA transcripts and the cytoskeleton, local translation of mRNA, and localisation to the axons (Donlin-Asp et al., 2017; Fallini et al., 2016, 2014, 2012). RNA binding proteins like HuD and IMP1 have been shown to play a role in the localisation of  $\beta$ -actin and growth-associated protein 43 (GAP-43) mRNA transcripts which are important for correct growth of axons (Fallini et al., 2014, 2011). Overall, the findings suggest that SMN plays an important role in the localisation and local translation of mRNAs with important axonal functions. The disruption of these functions through a significant decrease of SMN levels is likely to contribute to the axonal defects observed in SMA.

Closely associated with mRNA trafficking defects, a decrease of SMN protein has been linked to the impairment of the cytoskeleton. The cytoskeleton plays an important role in the neuronal architecture and function, including the formation of growth cones during development which are mediated by actin dynamics. The rho-associated kinase (ROCK) pathway is a key regulator of actin dynamics, and it has been shown that knocking down SMN leads to an upregulation of the ROCK pathway followed by inhibition of neuronal outgrowth (Bowerman et al., 2007). Further work demonstrated that an inhibition of the ROCK pathway with the inhibitors Y-27632 or Fasudil led to a prolonged life span, with Y-27632 partially ameliorating defects in NMJ maturation in an intermediate mouse model of SMA (Bowerman et al., 2010, 2012b). As the cytoskeleton is crucial for signalling and trafficking of mRNA within motor neurons, it is possible that an impairment of the cytoskeleton leads to an impairment of the local translation of mRNAs.

Furthermore, the diverse functions of the SMN protein have also been suggested to link to stress granule formation, translation, DNA repair, endocytosis, autophagy, mitochondrial homeostasis and ubiquitin pathways (as reviewed by Chaytow et al., 2018; Singh et al., 2017). Even though SMN is implicated in a variety of important functions, the actual disease-causative function of SMN remains unclear.

## Healthy individual



## Spinal muscular atrophy

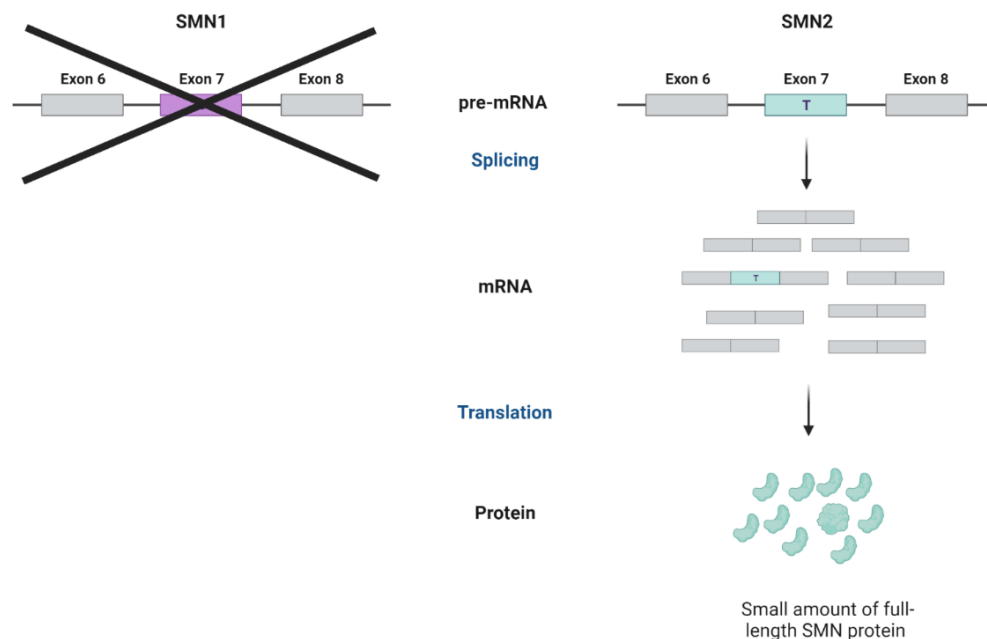


**Figure 1.1: C to T translation within exon 7 of the *SMN2* gene leads to alternative splicing and a reduction in produced functional full-length SMN protein.** The figure provides an overview of the genetics of spinal muscular atrophy. *SMN1* pre-mRNA is spliced to include exon 7, producing functional full-length SMN protein. A C to T translation within exon 7 of the *SMN2* gene causes a C to U alteration in pre-mRNA. This leads to an alternative splicing of the *SMN2* gene, thereby producing only around 10% of functional full-length SMN protein. The deletion of *SMN1* coupled with the resulting low levels of functional full-length SMN produced by the *SMN2* gene are causative of SMA. Figures based on Burghes and Beattie, 2009.

### **1.1.2. Clinical manifestation of SMA**

SMA is caused by deletions and mutations of the *SMN1* gene and is characterised by muscle weakness and atrophy, but the severity of the disease and the age of symptom onset can vary considerably. Some patients show very severe forms of the disease from infancy onwards, while others only develop symptoms during adulthood. There is a variety of reports, with the first one dating back to 1891, that detailed clinical manifestations, hallmarks of the disease and clinical severities in patients of different ages (Dubowitz, 2009). In 1991, the variety of previously described phenotypes was categorised into three different types of SMA at the International Consortium on Spinal Muscular Atrophy sponsored by the Muscular Dystrophy Association. The classifications were based on the highest achieved motor function of an individual with SMA and their age of onset (Munsat, 1991; Munsat and Davies, 1992). These classifications were subsequently modified to include a more severe type of the disease with a prenatal onset (Russman, 2007; Zerres and Davies, 1999) and are used to classify SMA patients until today. The recognised types of SMA range from type 0 to type 4 and are detailed below and in Table 1.1.

SMA type 0 is the most severe form of the disease (Dubowitz, 1999). Symptoms begin prenatally and are often associated with decreased foetal movements in the weeks prior to birth. After birth, the neonates present with severe weakness and may experience areflexia, facial diplegia, atrial septal defects, joint contractures and respiratory problems (Kolb and Kissel, 2015). Infants with type 0 typically require respiratory and feeding support. Most infants do not survive beyond 6 months of age. Typically, SMA type 0 infants only have one copy of the *SMN2* gene (Kolb and Kissel, 2015; Wadman et al., 2017).

SMA type 1 is a severe form of the disease that is also known as Werdnig-Hoffman disease, due to the first reports of patients presenting with this type of SMA from Guido Werdnig and Johan Hoffmann (Dubowitz, 2009). Infants are usually diagnosed in their first 6 months of life and they present with hypotonia, poor head control and reduced or absent tendon reflexes. Per

classification, SMA type 1 infants are never able to sit unassisted. They often develop tongue and swallowing weakness and as a result the infants are at risk of aspiration and a failure to thrive (Kolb and Kissel, 2015). Infants with type 1 SMA usually do not survive past 2 years of life due to the development of respiratory failure (Zerres and Rudnik-Schöneborn, 1995). This type of the disease is the most common type with 60% of all SMA patients presenting with type 1 (Ogino and Wilson, 2004). Typically, SMA type 1 infants have one to two, but a maximum of three *SMN2* gene copies (Kolb and Kissel, 2015; Wadman et al., 2017).

SMA type 2 is an intermediate form of the disease with a disease onset prior to 18 months of age. Per classification, children with SMA type 2 are never able to stand or walk unassisted and will require a wheelchair, although they might be able to sit unassisted. For this type of SMA the symptoms often start as delay or failure to meet motor milestones during the first 2 years of life. Children present with progressive weakness of the proximal legs that is more pronounced than weakness of the arms (Kolb and Kissel, 2015). Other symptoms include hypotonia and areflexia and often comorbidities such as muscular weakness, progressive scoliosis, joint contractures, and ankylosis of the mandible are present (Kolb and Kissel, 2015). Children with type 2 SMA usually survive past 2 years of life and typically have three copies of the *SMN2* gene (Kolb and Kissel, 2015; Wadman et al., 2017).

SMA type 3 is a mild form of the disease that is divided into two subgroups depending on the age of disease onset. SMA type 3a has a disease onset of between 18 months and 3 years of age, while SMA type 3b has a disease onset later than 3 years of age. Type 3 SMA is also referred to as Kugelberg-Welander disease. Per classification, patients with type 3 SMA are able to walk unassisted at some point during their life. However, they might lose this ability later on and develop limited mobility and the need to use a wheelchair. SMA type 3 patients present with progressive muscle weakness that is more pronounced in the legs than in the arms, but they are mostly spared a development of comorbidities such as scoliosis and respiratory muscle weakness (Kolb and Kissel, 2015). Life expectancy is not reduced and patients

with SMA type 3 usually have three or more copies of the *SMN2* gene (Kolb and Kissel, 2015; Wadman et al., 2017).

SMA type 4 is the mildest form of the disease with a disease onset that is typically in adulthood. Patients with type 4 SMA show similar symptoms to type 3 patients but with an even later disease onset, often only after the age of 30 years. As with type 3, patients are able to walk unassisted and generally only have mild motor impairments (Kolb and Kissel, 2015). Life expectancy is not reduced and patients with SMA type 4 usually have four to eight copies of the *SMN2* gene (Kolb and Kissel, 2015; Wadman et al., 2017).

**Table 1.1: Clinical types of Spinal muscular atrophy**

| Type | Age of Onset        | Survival  | Highest Function         | SMN2 copy number |
|------|---------------------|-----------|--------------------------|------------------|
| 0    | prenatal            | <6 months | never breath unsupported | 1                |
| 1    | <6 months           | <2 years  | never sit unsupported    | 1-2              |
| 2    | <18 months          | >2 years  | never stand unsupported  | 3                |
| 3    | >18 months          | adult     | stand/walk alone         | 3+               |
| 3a   | 18 months - 3 years | adult     | stand/walk alone         | 3+               |
| 3b   | >3 years            | adult     | stand/walk alone         | 3+               |
| 4    | adult               | adult     | stand/walk alone         | 4-8              |

### 1.1.3. Therapeutics in SMA

Since the discovery of the disease-causative mutation in the human *SMN* gene and the resulting decrease of full-length SMN levels, a variety of therapeutic strategies for SMA have been developed. The main focus of therapeutics research was to develop strategies that restore SMN protein production or increase full-length SMN protein levels in SMA patients. Strategies include the replacement of the mutated *SMN1* gene, or modification of the aberrant splicing of *SMN2* pre-mRNA through the use of antisense oligonucleotides (ASO) or small splicing-altering molecules. Currently, there are three SMA therapies that are approved by the FDA (Food and Drug Administration) in the United States and the EMA (European Medicines Agency) in Europe: Nusinersen (marketed as Spinraza by Biogen), onasemnogene abeparvovec (marketed as Zolgensma by Novartis), and risdiplam (marketed as Evrysdi by

Roche). For a detailed review of these approved therapies and further therapeutic strategies currently in clinical trial see reviews by Chaytow et al., 2021 and Shorrock et al., 2018.

Nusinersen (marketed as Spinraza by Biogen) was approved by the FDA in December 2016 and by the EMA in May 2017 (Table 1.2). Nusinersen is a splicing modifier of *SMN2* in form of an antisense oligonucleotide (ASO), that functions by manipulating the splicing pattern of *SMN2* to produce full-length SMN. ASOs are short, single stranded, synthetic oligodeoxynucleotides, binding to the pre-mRNA of *SMN2* to boost the inclusion of exon 7 in the *SMN2* gene during splicing. Nusinersen is approved for all ages and SMA patients of all types. It is administered via intrathecal administration, initially administering four loading doses followed by a maintenance dose every 4 months. Clinical trials showed that nusinersen was able to increase survival without permanent respiratory support in SMA type 1 patients, as well as improving development of motor functions and motor milestones in SMA patients with types 1-3 (as reviewed by Albrechtsen et al., 2020). Further, nusinersen treatment in pre-symptomatic SMA patients showed near-normal motor development, while motor function improvements in SMA type 2 and 3 were less evident (as reviewed by Albrechtsen et al., 2020).

The first gene replacement therapy, called onasemnogene abeparvovec (marketed as Zolgensma by Novartis; previously AVXS-101) was approved by the FDA in May 2019 and by the EMA in May 2020 (Table 1.2). Onasemnogene abeparvovec is a recombinant adeno-associated viral vector serotype 9 (AAV9) that delivers a functional copy of the *SMN1* gene. The AAV9 vector encodes human SMN protein under the control of the cytomegalovirus enhancer/chicken- $\beta$ -actin hybrid promoter. This gene therapy is approved for patients under 2 years of age that have a bi-allelic mutation of the *SMN1* gene and that have SMA type 1, or that possess less than three *SMN2* copies. Onasemnogene is administered as a single AAV9 delivery via an intravenous injection. Clinical trials assessed the efficacy and safety of delivering this treatment in pre-symptomatic SMA patients, patients under 6 months (with bi-allelic *SMN1* mutation type 1 SMA with 1 or 2 copies) and under 2 years of age

(with bi-allelic *SMN1* mutation type 1 SMA with 1 or 2 copies) and found the treatment to be effective for all groups (as reviewed by Blair, 2022). However, this therapy can lead to liver complications, including acute liver failure and acute serious liver injury (Chand et al., 2021). This gene therapy improved event-free survival, increased motor function and motor milestone outcomes, and patients were able to maintain these improvements up to a median of approximately 5 years (as reviewed by Blair, 2022). Further, an age-appropriate achievement of motor milestones and improvements in motor function were shown in pre-symptomatic SMA patients with three *SMN2* copies, indicating the benefit of early treatment with onasemnogene (as reviewed by Blair, 2022). While motor function was improved and some motor milestones were met in SMA patients with two *SMN2* copies following onasemnogene treatment, the outcome in this cohort was not age appropriate and some deficits persisted (as reviewed by Blair, 2022). Despite these promising observations, onasemnogene abeparvovec does not cure SMA and might induce hepatotoxicity in SMA patients.

Risdiplam (marketed as Evrysdi by Roche) was approved by the FDA in August 2020 and by the EMA in March 2021 (Table 1.2). Risdiplam is a small molecule therapy that targets the alternative splicing of *SMN2* pre-mRNA. It acts as a splicing modulator of *SMN2* by directly binding to *SMN2* pre-mRNA at two sites, stabilising the ribonucleoprotein complex and thereby promoting exon 7 inclusion and the production of full-length SMN protein. Risdiplam is delivered orally, taken once daily, and it is approved for patients of 2 months of age or older with all types of SMA (in the EU classified as: SMA type 1, 2 or 3 with 1-4 copies of *SMN2*). Clinical trials showed that risdiplam was able to significantly improve motor function in SMA type 1 patients and SMA types 2 or 3 (aged 2–25 years) patients, with patients being able to maintain these motor improvements up to 2 years while treated with risdiplam (as reviewed by Paik, 2022).

The above-described therapies are continuously undergoing clinical trials to maximise their effects on SMA patients of all types and ages. Initially, onasemnogene abeparvovec was used to treat patients with severe SMA type

1 who possess up to 2 *SMN2* copies in the START, STRIVE and SPRINT trials, where it proved to be safe for use in both symptomatic and pre-symptomatic infants (René and Parks, 2023). In January 2020, Novartis launched a program to treat all patients with SMA under the age of 2 years with onasemnogene abeparvovec. Nusinersen and risdiplam are approved for treatment of patients of all ages and types of SMA. As nusinersen was the first approved medication for SMA, it has been heavily utilised as stand-alone therapy for SMA patients of all types in the CHERISH, NURTURE and EMBRACE clinical trials. After the approval of onasemnogene abeparvovec, both nusinersen and risdiplam are predominantly given to patients with less severe forms of the disease that are not eligible for the gene therapy, or they are utilised as part of a combinational therapy regimen like in the RESPOND (Kuntz et al., 2023) and JEWELFISH (Chiriboga et al., 2023) clinical trials. Since all approved therapeutics are now widely being utilised as combinational therapies, the clinical trial approach and outcome measures have changed. While early clinical trials, like the START trial, were focused on assessing if the medications are able to extend the lifespan of patients with severe types of SMA (Mendell et al., 2021), the latest clinical trials, both stand-alone or combinational, are exploring the capacity of patients to reach new motor milestones, to improve their motor functions (often measured with the Hine section 2 motor milestone score), and to manage their respiration needs (Chiriboga et al., 2023; Kuntz et al., 2023; René and Parks, 2023). Clinical trials are now striving to provide the best possible combinational therapy approach for all types of SMA patients and *SMN2* copy numbers, and to improve unmet needs of patients after receiving previous therapy.

A number of other therapeutics are currently in different phases of clinical trials and are not yet approved by the authorities. Despite valuable therapeutic potential of the currently approved therapies, there are still downsides to these therapeutics that should not be disregarded. Both nusinersen and risdiplam need to be administered repeatedly, potentially causing strain on the patients, especially in the case of the repeated intrathecal delivery of nusinersen. Further, none of the developed therapies present a cure for the disease. Even in the case of the very promising gene therapy, it is not yet clear if a single

injection will yield a lifelong effect, or if subsequent doses will have to be administered once the initially treated patient cohort has reached adulthood. Despite the improvements that can be seen following the treatment with all three approved therapeutics, motor deficits remain in treated children and patients continue to present with complex deficits and needs (Audic et al., 2020; Lavie et al., 2021; Matesanz et al., 2019). For example, after one year of treatment with nusinersen, patients with SMA type 1 and 2 showed improvements in axial, proximal and distal motor function, but no improvements in standing, transfer or walking (Audic et al., 2020). Further, after two years of nusinersen treatment patients remained stable in their need for assisted ventilation and did not worsen. However, two years of nusinersen treatment were not able to improve respiratory needs of patients (Lavie et al., 2021). SMA patients treated with onasemnogene abeparvovec when they were more than 7 months old, were able to achieve some motor milestones, however these milestones were delayed and not age-appropriate, with some patients not being able to sit or stand, or only stand when assisted by a knee ankle foot orthosis (Matesanz et al., 2019). Other complementary therapeutic strategies will likely be needed to help against systemic consequences of SMN loss in patients, battling persisting deficits. Currently, an array of drugs that are already approved, or still in clinical trials for other neurodegenerative disorders, are under investigation for a potential therapeutic effect in SMA when combined with SMN replacement or increasing therapies (as reviewed by Chaytow et al., 2021). Despite the immense therapeutic advances in the field of SMA to date, further fundamental investigation of SMA pathogenesis and progression is needed to facilitate the identification of new potential targets that can in turn be utilised for therapeutic strategies. Potential stand-alone or combinational therapeutic strategies will be imperative to improve both, neuronal and non-neuronal, pathologies in SMA patients and eventually cure this devastating disease.

**Table 1.2: Approved therapeutics for the treatment of SMA**

| Drug                    | Market name | Company  | Mechanism                                                  | Targeted patients                                       | Administration route                                               |
|-------------------------|-------------|----------|------------------------------------------------------------|---------------------------------------------------------|--------------------------------------------------------------------|
| Nusinersen              | Spinraza    | Biogen   | antisense oligonucleotide splicing modifier of <i>SMN2</i> | all types of SMA                                        | intrathecal: 4 loading doses, then maintenance dose every 4 months |
| Onasemnogene ABEPRIVVEC | Zolgensma   | Novartis | gene replacement of <i>SMN1</i>                            | SMN type 1 patients patients with <3 <i>SMN2</i> copies | single dose of intravenous injection                               |
| Risdiplam               | Evrysdi     | Roche    | small molecule splicing modifier of <i>SMN2</i>            | patients >2 months of age                               | oral dose once daily                                               |

#### 1.1.4. Animal models of SMA

After the discovery of the causative genetic mutation responsible for SMA, the development of suitable animal models for the investigation of underlying cellular and molecular mechanisms of the disease started. Due to limited availability of suitable patient materials and post-mortem tissues at various stages throughout the disease time course (Monani and De Vivo, 2014), animal models of the disease play an imperative role in SMA research. SMA is a disease unique to humans, as the mutations affecting the splicing of *SMN2* are solely found in the human species (Rochette et al., 2001). Knockout and null mutations of the *Smn* gene in mice has been shown to be lethal (Schrack et al., 1997), therefore a range of genetic modifications has been performed to generate SMA-like disease models in various species, including invertebrate and vertebrate models.

*Drosophila* (*Drosophila melanogaster*), commonly known as fruit flies, have been utilised as model system for SMA. The *drosophila* genome only contains a single *smn* gene homologue, therefore point mutations or transposon insertions have been used to generate SMA-like *drosophila* disease models for knockout or knockdown studies (Chan et al., 2003; Rajendra et al., 2007). Similar to SMA patients, they exhibit comprised motor abilities and display defective axonal arborisation in motor neurons (as reviewed by Edens et al., 2015). *Drosophila* models generally have several benefits that make them an interesting model organism for neurodegenerative disease research. These include a short life cycle for easy and inexpensive maintenance of colonies, a rapid reproduction cycle with large numbers of progeny, striking organisational similarities with the vertebrate brain but with a reduced complexity in

comparison to the mammalian brain, and a reduced genome complexity (McGurk et al., 2015). *Drosophila* demonstrate a valuable model for genetic mapping and for conducting easy and speedy screens of genetic modifiers. It was shown that *drosophila* could be used to identify *smn*-interacting genes of the BMP signalling pathway, genes involved in endocytosis and RNA processing, and *smn*-interacting genes that are RNA splicing factors (as reviewed by Edens et al., 2015).

Another widely used SMA-like model system is the zebrafish (*Danio rerio*). The zebrafish genome only contains one *smn* gene homologue, therefore knockdown of expression through antisense oligonucleotides has been used to generate SMA-like zebrafish disease models (McWhorter et al., 2003). Zebrafish demonstrate an excellent model organism for neuromuscular defects and MND due to their simple neuromuscular organisation, and their well-characterised motor neurons and NMJs (as reviewed by Edens et al., 2015). Further, zebrafish can be easily genetically manipulated and their embryos offer optical transparency, thereby offering valuable imaging opportunities *in vivo*. It was shown that the administration of antisense oligonucleotides into zebrafish embryos can inhibit the translation of target mRNA, which in turn could reduce Smn protein by over 60%, and resulted in abnormal axonal morphology (McWhorter et al., 2003).

Animal models like zebrafish, *drosophila* and other invertebrate models like *Caenorhabditis elegans* can be utilised to yield valuable insights into disease pathology and molecular mechanisms by offering a platform for easy and quick large-scale screens and testing of genetic modifiers. However, caution needs to be exercised as some findings from invertebrate and non-mammalian vertebrate models contradict findings from mammalian disease models (Burghes and Beattie, 2009). One example for this is that expression of SMN in muscle alone has little impact on SMA mice, while expressing SMN in muscle of *drosophila* leads to a substantial rescue of SMN deficient *drosophila* animals (as reviewed by Burghes and Beattie, 2009). These findings demonstrate that conclusions about the human disease mechanisms and phenotype cannot always be drawn from these relatively basic animal models.

As humans and mice are more closely conserved than invertebrates or non-mammalian vertebrates, a number of SMA-like mouse (*Mus musculus*) models were generated. As other species, mice only possess one single *Smn* gene that almost exclusively produces full-length Smn protein, which poses a problem when trying to recapitulate the human SMA phenotype. A range of transgenic approaches has therefore been taken to generate SMA-like mouse models (as reviewed by Bebee et al., 2012). The first mouse model was developed by disrupting exon 2 or exon 7 of the murine *Smn* gene, leading to a homozygous knockout of *Smn* (*Smn*<sup>-/-</sup>) with a complete lack of Smn protein (Schrack et al., 1997). This *Smn*<sup>-/-</sup> mouse model proved to be embryonically lethal at the 8-cell stage of development (Schrack et al., 1997). In contrast, it was shown that a heterozygous disruption in exon 2 of murine *Smn* (*Smn*<sup>+/-</sup>) leads to the production of a mouse model with a normal lifespan and a normal appearance (Jablonka et al., 2000). Following these findings, transgenic methods of supplementing human *SMN2* to a null murine *Smn* background were utilised to manipulate the dosage of produced Smn protein in mice and therefore mimic a human SMA phenotype.

The introduction of two copies of the human *SMN2* transgene onto a murine *Smn* null background, led to the development of a new SMA mouse model (Figure 1.2). This *Smn*<sup>-/-</sup>;*SMN2* mouse model is based on a homozygous disruption in exon 2 of *Smn* leading to a complete knockout of murine *Smn* (Monani et al., 2000). With two copies of human *SMN2* this model displays a very severe phenotype of the disease with a lifespan of approximately 6 days. However, the introduction of eight instead of two *SMN2* copies on a null *Smn* background leads to phenotypically normal mice with a normal lifespan, thereby demonstrating the dose dependent requirement of Smn (Monani et al., 2000).

The 'Taiwanese' mouse model of SMA shows genetic similarity to the *Smn*<sup>-/-</sup>;*SMN2* mouse model. In this model, varying copy numbers of human *SMN2* are induced onto a null *Smn* background (Figure 1.2). Here, however, the *Smn* null background is produced by a homozygous disruption in exon 7 rather than exon 2, leading to the development of the *Smn*<sup>-/-</sup>;*SMN2*(2*Hung*) or 'Taiwanese'

mouse model (Hsieh-Li et al., 2000). This mouse model is also a severe mouse model of SMA, but it has a varying lifespan depending on its copy number of *SMN2* transgenes. With the expression of fewer *SMN2* copies, these mice have a mean survival of about 10 days, while they show a normal lifespan when they possess a higher copy number of *SMN2* transgenes (Hsieh-Li et al., 2000).

The *Smn*<sup>-/-</sup>;*SMN2* mouse model was the basis for the development of yet another mouse model of SMA. Here, an additional transgene was induced onto a null murine *Smn* background. The *SMN*Δ7 transgene was developed from human *SMN* gene cDNA that lacked exon 7 (*SMN*Δ7) under the control of the human *SMN2* promoter, as it would be the case in most transcripts produced by *SMN2* (Figure 1.2). Combining the *Smn*<sup>-/-</sup>;*SMN2* mouse model with the *SMN*Δ7 transgene led to the development of the severe *Smn*<sup>-/-</sup>;*SMN2*;*SMN*Δ7<sup>+/+</sup> mouse model (known as the *SMN*Δ7 mouse) of SMA (Le et al., 2005). The addition of this transgene to the *Smn*<sup>-/-</sup>;*SMN2* mouse model, extended their life expectancy from approximately 6 to 13 days (Le et al., 2005). This severe SMA mouse model recapitulates the human disease phenotype fairly well by showing weight loss and motor deficits due to muscular atrophy and motor neuron loss (Le et al., 2005). The *SMN*Δ7 mouse model is possibly the most heavily used mouse model of SMA, as it allows to study the severe characteristics of the disease over a prolonged time frame compared to other severe mouse models of the disease.

While all severe mouse models of SMA have inserted copies of the human *SMN2* transgene and a complete knockout of murine *Smn*, a recent development generated a mouse model of SMA through genetic modification of the murine *Smn* gene (Figure 1.2). The *Smn*2B allele was designed as replacement vector and induced into the endogenous *Smn* locus through homologous recombination. This point mutation alters the central part of the exon splice enhancer region through a substitution of 3 nucleotides (GGA to TTT) within exon 7. The positive selection cassette *pgk-neo* was then inserted for this replacement vector into the unique *Bam*HI site, ~180 bp distal to exon 7 (Hammond et al., 2010). This mutation promotes the exclusion of exon 7 and

the production of the Smn $\Delta$ 7 protein, thereby replicating the splicing pattern of the human *SMN2* gene (DiDonato et al., 2001; Hammond et al., 2010). The induction of this point mutation into the splice enhancer site in exon 7 of the murine *Smn* gene led to the generation of the *Smn*<sup>2B/-</sup> mouse model, where it leads to reduced levels of full-length Smn protein, mimicking the effects of human *SMN2* (Bowerman et al., 2012a; DiDonato et al., 2001; Hammond et al., 2010). The *Smn*<sup>2B/-</sup> mouse model expresses approximately 15% full-length Smn protein, with this lack of murine Smn disrupting motor neuron function and leading to progressive loss of lower motor neurons (Bowerman et al., 2012a). It is an intermediate model of the disease with a lifespan that was originally described as 28 days (Bowerman et al., 2012a). In our hands, this mouse model has a life expectancy of 18 days, with onset of symptoms around P10. The comparatively long lifespan of the *Smn*<sup>2B/-</sup> mouse model and the late symptomatic onset offer an extended pre-symptomatic time window that cannot be seen in other mouse models of SMA, thereby facilitating the study of the temporal and molecular pathological events that result in motor neuron death in this mouse model. Our and other laboratories observed phenotypic characteristics including a smaller body size with smaller ears and a smaller tail, weight loss, and motor deficits and muscle weakness compared to control littermates (Bowerman et al., 2012a; Courtney et al., 2019).

As shown in Figure 1.2, the four above-described mouse models of SMA do not just have unique genetic characteristics, but these genetic models were also bred on different genetic backgrounds. While the induced genetic manipulation is a strong determinant of the displayed severity and phenotype of each of those models, the effect that the genetic background of these mice has on phenotype and severity, cannot be underestimated. A previous study explored the effects of genetic background on the *Smn*<sup>2B/-</sup> mouse model and found profound differences on phenotype and disease course between *Smn*<sup>2B/-</sup> mice on a BL6 and a FVB background (Eshraghi et al., 2016). While *Smn*<sup>2B/-</sup> mice did not exhibit different Smn protein levels between a BL6 and a FVB background, *Smn*<sup>2B/-</sup> mice on a FVB background showed significantly reduced weight, earlier reduced muscle strength, earlier reduced muscle fibre cross-sectional area, earlier motor neuron loss and earlier neuromuscular junction

pathology (Eshraghi et al., 2016). Similarly, it has been shown that SMN $\Delta$ 7 mice have a much shorter life span if bred on a BL6 instead of a FVB background (Le et al., 2005) and that the Taiwanese mice showed an increased survival when backcrossed on the BL6 background instead of the original FVB background (Ackermann et al., 2013), making genetic background an important factor in time course and severity of disease.

All of the above discussed mouse models come with some advantages and disadvantages for the investigation of specific aspects of SMA research. The *Smn*<sup>-/-</sup>; *SMN2* mouse model only has a life span of about 5 days (Monani et al., 2000) and is a good representation of the severe type 1 SMA (Edens et al., 2015). The Taiwanese model has a mean lifespan of about 10 days (Hsieh-Li et al., 2000) and the SMN $\Delta$ 7 has a lifespan of up to 13 days (Le et al., 2005), making these two models good representations of type 2 SMA (Edens et al., 2015). Some of the advantages for all these models, are rooted in the fact that they have all been genetically altered to include copies of the human *SMN2* transgene. These models are especially important for studies that investigate the effects on phenotype and neuropathology of therapeutics that alter the splicing of *SMN2*, as well as allowing the investigation of new combinational therapies with already approved *SMN2* splicing compounds. However, a potential disadvantage of these models is their short lifespan and their early disease onset, often prohibiting studies that want to investigate pre-symptomatic aspects of the disease or time course analysis of degeneration. The *Smn*<sup>2B/-</sup> mouse model on the other hand, does not possess a human transgene, but a point mutation in the murine *Smn* gene that mimics the splicing pattern of human *SMN2* (Bowerman et al., 2012a). This intermediate mouse model is a representation of a less severe type of SMA with its life span of up to 28 days and disease onset around P10. In contrast to other SMA mouse models, this model has the advantage of a long pre-symptomatic phase and an extended symptomatic phase, making it a useful model for the investigation of pre-symptomatic aspects, time course analysis and the investigation of therapeutic strategies that do not rely on *SMN2* splicing alterations.

In addition to the above discussed mouse models with systemic Smn depletion, an array of cell-type specific mouse models has been developed to determine the specific effect of Smn depletion when only specific cell types exhibit low Smn levels (Bebbee et al., 2012). Tissue specific models have facilitated the investigation of Smn depletion in neuronal cells (Cifuentes-Diaz et al., 2002), muscle cells (Cifuentes-Diaz et al., 2001), liver cells (Vitte et al., 2004) and spinal cord motor neuron progenitors (Park et al., 2010), and have increased the potential for studies on disease mechanisms and studies on effects of therapeutics in specific tissues (Bebbee et al., 2012). Furthermore, a range of other SMA-like mouse models have been created through different transgenic approaches, each of them presenting different benefits for the investigation of specific aspects of the disease (as reviewed by Bebbie et al., 2012 and Edens et al., 2015). The variety of available mouse models of the disease provides the opportunity to strengthen findings, as well as observe and compare them in models of different severities and genetic backgrounds, ultimately facilitating to gain deeper insights into the underlying cellular and molecular mechanisms of the disease.

|                                | <i>Smn</i> <sup>-/-</sup> ; <i>SMN2</i> | <i>Smn</i> <sup>-/-</sup> ; <i>SMN2</i> (2Hung) | <i>Smn</i> <sup>-/-</sup> ; <i>SMN2</i> ; <i>SMN</i> Δ7 <sup>+/+</sup> | <i>Smn</i> <sup>2B/-</sup>                                                                           |
|--------------------------------|-----------------------------------------|-------------------------------------------------|------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------|
| Known as                       | severe model                            | Taiwanese model                                 | SMNΔ7 model                                                            | <i>Smn</i> <sup>2B/-</sup> model                                                                     |
| Murine <i>Smn</i>              | no                                      | no                                              | no                                                                     | yes                                                                                                  |
| Human copy of <i>SMN2</i>      | yes<br>2 copies                         | yes<br>2 copies                                 | yes<br>2 copies                                                        | no                                                                                                   |
| Other genetics                 |                                         |                                                 | Transgenic human <i>SMN2</i><br>copy lacking exon 7 (Δ7)               | modified <i>Smn</i> 2B allele of <i>Smn</i> :<br>mutation of exonic enhancer<br>in <i>Smn</i> exon 7 |
| Original genetic<br>background | C57BL/6J                                | FVB/N                                           | FVB/N                                                                  | C57BL/6 x CD1 hybrid                                                                                 |
| Reference                      | Monani et al., 2000                     | Hsieh-Li et al., 2000                           | Le et al., 2005                                                        | Bowerman et al., 2012                                                                                |

**Figure 1.2: Genetics of mouse models of spinal muscular atrophy.** This figure provides an overview of the genetics of the four most commonly utilised mouse models of SMA. While the three severe mouse models of SMA - severe model, Taiwanese model and SMNΔ7 model – are all based on a null murine *Smn* background with induced copies of human *SMN2* transgenes, the intermediate *Smn*<sup>2B/-</sup> mouse model is generated through the modification of the *Smn*2B allele of the murine *Smn* gene. Images of phenotypic appearance of mice are taken from the studies that first described the respective model and are labelled to highlight SMA and control mice in each model.

## 1.2. Pathology in SMA

### 1.2.1. Motor neuron pathology in SMA

SMA is characterised by the degeneration of lower alpha motor neurons in the brainstem and spinal cord. Lower motor neurons are elongated neuronal cells that connect the brainstem or spinal cord with the associated muscle. They are composed of the cell body (soma) located in the cranial nerve nuclei of the brainstem or the ventral horn of the spinal cord, the axon, which extends from the cell body in the spinal cord to the periphery of the associated muscle, and the neuromuscular junction (NMJ), which connects the pre-synaptic terminal of the motor neuron with the post-synaptic motor endplate of the muscle. Pathological features of each of these components of the motor neuron have previously been reported in SMA, making motor neuron and NMJ deficits important hallmarks of the disease.

#### 1.2.1.1. Causes for motor neuron susceptibility in SMA

It has long been established that SMA is caused by a reduction of full-length SMN protein levels. However, it remains largely unclear why reduced levels of SMN protein lead to a selective susceptibility of motor neurons, especially since SMN protein is ubiquitously expressed. This preferential susceptibility of the motor system in SMA, raises questions about the temporal and spatial requirements of SMN protein. Theories for possible reasons of the motor neuron susceptibility in SMA can be found in the role of SMN protein. It has been demonstrated that high levels of SMN are required in the motor neurons during the embryonic and early postnatal phases in order to enable a correct development of the motor system (Le et al., 2011; Lutz et al., 2011). Ubiquitously increased SMN levels during early postnatal development, but not later than one week post-natal, rescued the SMA-like phenotype of a severe SMA mouse model (Le et al., 2011; Lutz et al., 2011). This requirement of high SMN levels during this early postnatal development, correlates with the maturation of the motor system in mice (Tisdale and Pellizzoni, 2015).

The idea that motor neurons have a higher threshold requirement for SMN is supported by recent findings. A study demonstrated that the SMN expression levels varied significantly between different tissues and ages of control and SMA mice in mouse models of SMA (Groen et al., 2018). SMN levels of healthy control mice significantly increased in the brain and spinal cord in comparison to non-neuronal tissues within the first week of postnatal development (between P2 and P8), demonstrating that the requirements for SMN seem to vary between nervous tissues and non-neuronal tissues in healthy development and maturation (Groen et al., 2018). In stark contrast, SMN levels decreased in the brain and spinal cord in SMA mice during the first postnatal week, emphasising the large effect that SMN depletion has on the nervous tissue (Groen et al., 2018).

Another theory as to why motor neurons are more susceptible than other tissues following SMN depletion, is the idea that decreased SMN levels lead to a disruption in localisation and local translation of mRNAs (see section 1.1.1. for detailed description). This contributes to the axonal defects observed in

SMA (Donlin-Asp et al., 2017; Fallini et al., 2016, 2014, 2012). The very long axons of motor neurons are likely to have an increased requirement for local translation at their synapses, which can no longer be sustained due to the impaired local translation process (Donlin-Asp et al., 2017; Fallini et al., 2016).

#### 1.2.1.2. Motor neuron defects in SMA

Despite the unknown reasons for the increased susceptibility of the motor system following SMN depletion, this depletion results in several defects of the motor neuron that have been characterised in SMA patients and mouse models of the disease. The loss of lower alpha motor neurons is perhaps the most well-known hallmark of SMA. A reduction in the total number of motor neuron cell bodies has been shown in both SMA patients and SMA-like mouse models. A study conducted on post-mortem spinal cord tissue compared motor neuron numbers in control and SMA fetuses, as well as in post-mortem spinal cords from control and SMA type 1 neonates (Soler-Botija et al., 2002). They showed that SMA fetuses had a lower number of motor neurons in their spinal cords compared to their controls, and they further reported a 50-60% reduction of the number of motor neurons in neonates with SMA type 1 compared to their controls (Soler-Botija et al., 2002). Motor neuron loss is also a feature that is very prominent in mouse models of SMA, although the degree of motor neuron loss varies. In the severe *Smn*<sup>-/-</sup>; *SMN2* mouse model, motor neuron loss was not observed at P1, however by P5 (disease end-stage) there was an approximate loss of 35% in the lumbar L1-L6 spinal motor neurons and a 40% loss of facial motor neurons present in comparison to their control littermates (Monani et al., 2000). The *SMN*Δ7 mouse model showed no significant difference in lumbar L3-L6 spinal cord motor neuron number at P4, but a motor neuron number decrease of around 20% at P9 (Le et al., 2005). Another study demonstrated a 30% loss of cervical C3-C5 spinal motor neurons at P13 in *SMN*Δ7 mice compared to their controls (d'Errico et al., 2013). Further, it was shown that no significant loss of SMA motor neurons was present at P0, but a ~40%, 50% and 60% reduction of motor neurons was shown at P4, P9 and P13 respectively in lumbar L1 spinal motor neurons in *SMN*Δ7 mice compared to their controls (Mentis et al., 2011). They also showed that medial (innervating axial muscles) and lateral (innervating distal limb muscles) lumbar

L5 spinal motor neurons were differentially affected in SMN $\Delta$ 7 mice, with no significant loss of medial or lateral motor neurons at P4, a 35% and 50% loss of medial motor neurons at P9 and P13 respectively, but no significant loss of lateral motor neurons at P9 or P13 (Mentis et al., 2011). Quantifications of motor neuron numbers in the thoracic T9 and lumbar L1 and L5 spinal regions at P11 did not show any significant loss of motor neurons in the Taiwanese mouse model (Buettner et al., 2021). The *Smn*<sup>2B/-</sup> mouse model showed a ~50% decrease in the thoracic spinal motor neuron number at P15 in comparison to their controls (Courtney et al., 2019), a significant decrease in lumbar L1-L2 spinal motor neuron numbers at disease end-stage (Bowerman et al., 2012a; Buettner et al., 2021), but no significant decrease of lumbar L5 motor neurons in comparison to their controls (Buettner et al., 2021; Carlini et al., 2022).

A further motor neuron defect that has previously been observed in SMA mouse models, is a reduction of the motor neuron size associated with disease progression. Previous work showed that motor neurons were significantly smaller in the SMN $\Delta$ 7 mouse model at both P4 and P13, indicating that motor neurons atrophy might precede degeneration and loss of motor neurons (d'Errico et al., 2013). Further, work demonstrated that motor neurons showed a reduction in area size at P15 in *Smn*<sup>2B/-</sup> mice compared to their control littermates, but no significant difference in motor neuron size was observed at earlier P1, P5 or P10 time points (Courtney et al., 2019).

Furthermore, dysfunctions in the spinal sensory-motor circuit have been reported in several mouse models of SMA. Previous work demonstrated that a reduction of proprioceptive synapses can be observed early in the disease in the Taiwanese, SMN $\Delta$ 7 and *Smn*<sup>2B/-</sup> mouse model (Buettner et al., 2021; Carlini et al., 2022; Ling et al., 2010; Mentis et al., 2011). The Taiwanese and *Smn*<sup>2B/-</sup> mouse model exhibited a ~50% decrease in proprioceptive synapses at disease end stage (Buettner et al., 2021; Carlini et al., 2022), while the SMN $\Delta$ 7 mice exhibited a ~50% decrease at P4 and a ~70% decrease at disease end-stage (Buettner et al., 2021). These findings suggest that the loss of proprioceptive synapses on motor neurons is an important hallmark of SMA.

Defects of the motor system in SMA can also manifest in the axons of motor neurons. Axons extend from the ventral roots of the spinal cord to connect the soma with the motor endplate. A number of studies in SMA patients showed that the disease causes a reduction in the number of myelinated axons from the ventral roots of these patients (as reviewed by Pérez-García et al., 2017). Similar to SMA patients, a significant 78% reduction of myelinated axons from the ventral roots was shown in a mouse model of SMA (Cifuentes-Díaz et al., 2002).

### **1.2.2. Neuromuscular junctions and their involvement in SMA pathology**

#### **1.2.2.1. The neuromuscular junction**

The neuromuscular junction is a synapse that connects the axon of a lower motor neuron to a muscle fibre of a skeletal muscle, thereby facilitating an effective muscle contraction. The NMJ is made up of two main cellular parts, the pre-synaptic and post-synaptic part. The pre-synaptic part of the NMJ, also called the pre-synaptic motor nerve terminal, is formed by the distal end of a lower motor neuron, while the post-synaptic aspect is part of a skeletal muscle fibre. Both main cellular parts of the NMJ are surrounded and supported by glial cells, including myelinating and terminal Schwann cells, to support the motor neuron and NMJ during their development, for maintenance of the NMJ and to support synaptic function. The NMJ is the last point of connection between nervous system and skeletal muscle and is therefore imperative for functioning movement (Boyd and Gillingwater, 2017).

NMJ's are formed when axons from lower motor neurons grow out from the spinal cord and grow into the periphery to locate and reach their target muscle fibre. The target muscle fibre can be located because of a systematic mapping of the rostrocaudal axis of the motor pool onto the rostrocaudal axis of the muscle. This mapping is associated with the segmental ordering of axons in the nerve and is suggested to be aided by positional labels within the muscle and by axonal guidance at branch points in the nerve (Laskowski and Sanes, 1987). This developmental connection process happens quite early during embryonic development in mammals, namely already from embryonic day 12.5 in mice (Lin et al., 2001). Once the axon has reached the target skeletal

muscle fibre, a NMJ is formed and a series of steps towards maturation of the NMJ starts. During maturation, the NMJs undergo large morphological and functional changes that involve their pre- and post-synaptic aspects. At birth, each NMJ has multiple axonal inputs, making the NMJ polyinnervated. During the early postnatal development, excess synapses are removed until just one nerve terminal remains at each NMJ, a process known as 'synapse elimination' (Lichtman and Colman, 2000; Walsh and Lichtman, 2003). It is then ensured that this single NMJ expresses the correct synaptic proteins required for maintenance of a stable NMJ (Lichtman and Colman, 2000). During perinatal maturation, the post-synaptic muscle fibre also undergoes changes, involving an increase in number, clusters, and stability of the acetylcholine receptors (AChRs) at the motor endplate (Boyd and Gillingwater, 2017; Lin et al., 2001).

The goal of all these maturation steps is to build a stable and highly efficient NMJ, ensuring that neuromuscular transmission is effective and that it leads to reliable muscle contraction. To produce a muscle contraction, calcium channels are activated in the motor neuron through an action potential that arrives at the nerve terminal. This leads to an influx of calcium, thereby triggering synaptic vesicles to fuse with the pre-synaptic membrane and the neurotransmitter acetylcholine (ACh) gets released into the synaptic cleft between the neuron and the muscle. ACh then spreads across the synaptic cleft and binds to the AChRs clusters that are located on the motor endplate on the post-synaptic muscle fibre. The neurotransmitter binding causes a depolarisation and opens ligand-gated sodium channels, allowing that sodium ions enter the muscle. This then generates an action potential which triggers the muscle to contract (Hughes et al., 2006; Wood and R. Slater, 2001).

#### 1.2.2.2. Neuromuscular junction defects in SMA

Besides loss of motor neuron cell bodies, SMA pathology also affects the distal ends of the motor neurons, the neuromuscular junctions (NMJs). NMJs have been shown to be early and severe pathological targets in mouse models of SMA and present a hallmark of the disease with their variety of associated defects (Murray et al., 2008). These defects include presynaptic swelling and neurofilament accumulation, a reduced vesicle content, impairment in synaptic

transmission, and impaired motor endplate maturation (Kariya et al., 2008; Kong et al., 2009; Lee et al., 2011; Ling et al., 2010; Murray et al., 2008). As SMA is thought to be a die-back pathology, where motor neuron degeneration happens in a distal to proximal manner (Courtney et al., 2019; Murray et al., 2008), the perhaps most frequently observed defect of the NMJ is the progressive denervation of motor endplates. Here, the pre-synaptic terminal from the motor neuron axons gradually withdraws from the motor endplate. The distal end of the nerve terminal thereby forms a bulb, which is known as retraction bulb, leaving the motor endplate without innervation (Gillingwater and Ribchester, 2003; Murray et al., 2008). This progressive denervation has been demonstrated in numerous studies of different mouse models of SMA, showing evidence of motor endplates that remain fully innervated, ones that are only partially innervated, as well as evidence of vacant endplates that have lost all their innervation from the pre-synaptic terminal (Buettner et al., 2021; Comley et al., 2016; Courtney et al., 2019; Lin et al., 2016; Ling et al., 2012; Mole et al., 2020; Murray et al., 2015, 2013, 2008; Thomson et al., 2012). The progressive retraction of the pre-synaptic terminal from the motor endplate, leaves them denervated before the pathology reaches the motor neuron cell bodies (Courtney et al., 2019; Murray et al., 2008).

A further well-documented defect of the NMJ is the presence of pre-synaptic swelling due to a substantial amount of neurofilament accumulation (NFA) in the distal axons and motor nerve terminals. Neurofilaments are the intermediate filaments of neurons and are a component of the cytoskeleton that is produced within the cell body of the motor neurons. Neurofilament is then transported into and along their axons where it is needed during development, in nerve conduction, and for axon diameter (Yuan et al., 2012). NFA in motor nerve terminals is observed in both patients and SMA mouse models, where it is seen located at the pre-synapse in various severities (Cifuentes-Diaz et al., 2002; Kariya et al., 2008; Kong et al., 2009; Murray et al., 2008). NFA has been repeatedly documented as a consequence of SMN depletion (Kariya et al., 2008; Kong et al., 2009; Murray et al., 2008). It has further been suggested that the observed NFA in SMA may be caused by an impairment of anterograde and retrograde axonal transport functions that are

associated with the depletion of SMN protein in SMA (Boyd and Gillingwater, 2017). In contrast, NFA has previously been reported to not be due to axonal transport defects, but rather to synaptic alterations (Dale et al., 2011). It therefore remains to be fully established how the degeneration of the NMJ and the onset of NFA relate to each other and whether NFA represents a consequence or primary cause of neuromuscular pathology (Boyd and Gillingwater, 2017; Cifuentes-Diaz et al., 2002).

NMJ defects are also associated with defects in the post-synaptic motor endplates that is formed by acetylcholine receptors (AChRs) on the muscle fibres of the associated muscles. These problems arise from deficits in post-natal maturation of the NMJ in SMA. During post-natal development, the AChRs alter their morphology from an immature plaque-like shape to a mature pretzel-like structure through reordering and perforation. In a SMA mouse model, a delay of this maturation process from plaque to pretzel-like shape has been observed (Biondi et al., 2008). The persisting of immature endplates has also been observed in the SMN $\Delta$ 7 mouse model of SMA (Kariya et al., 2008; Lee et al., 2011). They showed that at disease end-stage, there were significantly more immature endplates present in SMN $\Delta$ 7 mice in comparison to their controls (Kariya et al., 2008; Lee et al., 2011). The physiological maturation of motor endplates is further characterised by the change in expression from fetal  $\gamma$ -AChR subunits to adult  $\epsilon$ -AChR subunits during the first two post-natal weeks of development. The observed delay in the maturation process of NMJs in SMA is further supported by the delayed  $\gamma$ - to  $\epsilon$ -subunit exchange in post-natal endplates (Kariya et al., 2008; Kong et al., 2009). Studies showed that there was an increase in motor endplates expressing the fetal  $\gamma$ -AChR subunit instead of the adult  $\epsilon$ -AChR subunit in SMN $\Delta$ 7 mice in comparison to their controls (Kariya et al., 2008; Kong et al., 2009).

When NMJs are subjected to denervation and muscle fibres denervate under non-pathological conditions, then neighbouring motor neurons send out new axonal branches to re-innervate the muscle fibre (Slack et al., 1983). This is called sprouting. Two different types of sprouting have been described:

terminal sprouting, where sprouts extend from the pre-synaptic terminal of an innervated NMJ, and nodal sprouting, where sprouts extend from the internodes of axons (Hoffman, 1950). It has previously been shown that sprouting is a feature that can be observed in SMA patients (Crawford and Pardo, 1996) and mouse models of the disease (Comley et al., 2022; Murray et al., 2013). In the *Smn*<sup>2B/-</sup> mouse model sprouting was observed, but it was shown that the level of paralysis induced sprouting was decreased even though the rate of axon degeneration was not affected by the reduced levels of Smn in this model (Murray et al., 2013). Electromyography (EMG) studies in SMA patients have shown that patients with severe types of the disease have ongoing denervation, while patients with milder types of SMA possess larger motor units which suggests the presence of sprouting (Crawford and Pardo, 1996). Similar results of increased motor unit size and thereby indicative of collateral sprouting, were also seen in a longitudinal EMG study on SMA type 2 and 3 patients (Kang et al., 2014). The above results in SMA patients and mouse models suggest that sprouting could be a disease modifier in SMA.

All of the above-described motor neuron cell body, axon, and NMJ defects present hallmarks of SMA, but the question of their relation to each other remains largely unanswered. It has been proposed that the degeneration of the NMJs is causative for the loss and death of motor neurons. However, despite reports that NMJ loss precedes a quantifiable loss of motor neurons, the loss of NMJs has been shown to coincide with the onset of cell death pathways at the soma (Buettner et al., 2021; Courtney et al., 2019). A number of studies have demonstrated a strong activation of the P53 signalling pathway at pre-symptomatic stages in the spinal cord of the SMN $\Delta$ 7 and *Smn*<sup>2B/-</sup> mouse models of SMA, thereby showing that the activation of this cell death pathway precedes motor neuron loss (Buettner et al., 2021; Murray et al., 2015; Simon et al., 2017). These findings indicate that motor neuron death might be initiated prior to the visible and quantifiable loss and pathology of motor neurons in SMA, suggesting that the disease might not be a die-back pathology after all.

### **1.2.3. Other tissue defects in SMA and insights from patient studies**

As described in the previous sections, motor neuron loss and NMJ defects are hallmarks of SMA in both patients and mouse models of the disease. However, as reviewed by Abati et al., 2020, studies on animal and cellular models of SMA have shown that a correct functionality and the survival of motor neurons in SMA is dependent on a variety of other cell types, especially on glial cells, which are the most abundant cell type in the CNS. Glial cells surround motor neurons and are responsible for various functions, including for neuronal trophic support, remodelling of synapses, and for immune surveillance. It has been reported in post-mortem SMA patient studies that gliosis is associated with areas where motor neuron degeneration is present and where a large reduction in motor neuron number was found in the brain stem and spinal cord (Araki et al., 2003; Kuru et al., 2009). Further, astrogliosis was also reported to be present at the level of the spinal cord in a post-mortem study on a SMA type 1 patient (Rindt et al., 2015).

While SMA is characterised by the degeneration of motor neurons and the atrophy of associated muscles and is therefore classified as motor neuron disease, there have been many reports from patients and mouse models detailing pathologic changes in non-neuronal tissues, suggesting that SMN depletion also plays a role in non-neuronal tissues. In SMA patients, pathological changes have been shown in various non-neuronal tissues, including in pancreas (Bowerman et al., 2012c), spleen (Thomson et al., 2017), and in vasculature (Somers et al., 2016). Furthermore, there is also an array of associated heart defects that was reported in patients with SMA (as review by Wijngaarde et al., 2017). Similar defects in non-neuronal tissues have also been shown in mouse models of SMA, including in liver (Szunyogova et al., 2016), pancreas (Bowerman et al., 2014), spleen (Thomson et al., 2017), and vasculature (Somers et al., 2016). Additionally, skeletal muscle is implicated in SMA pathology, not just as a consequence of loss of innervation after motor neuron degeneration, but there has also been evidence that reduced SMN levels cause intrinsic defects of skeletal muscle in SMA patients and mouse models (as reviewed by Boyer et al., 2013). A previous study showed a delayed maturation of myotubes in SMA type 1 fetuses (Martínez-Hernández

et al., 2009). Further, skeletal muscle presents with an immature phenotype in both SMA patients and mouse models, when compared with that of age-matched controls (Boyer et al., 2014; Kariya et al., 2008; Kong et al., 2009). Further, a proteomic analysis in a SMA mouse model revealed abnormalities in the regulation of proteins involved in muscle function and pathology (Mutsaers et al., 2011).

While some pathological features of neuronal and non-neuronal tissues have been documented in SMA patients, most pathological features of the disease have been more extensively investigated in animal models of the disease. Although the investigation of pathological features in post-mortem tissues is of high importance to inform on disease mechanisms and help define suitable targets for therapeutic strategies, a sparse availability of suitable patient materials and post-mortem tissues at various stages throughout the disease time course often limits studies on patient tissues (Monani and De Vivo, 2014). Most autopsy studies are concentrated on a single individual with a single investigated tissue, and are conducted predominantly on SMA type 1 patients as there was a naturally higher mortality of this patient cohort compared to SMA patients with less severe types of the disease (Crawford and Pardo, 1996). However, there is a small number of reports detailing neuropathology in SMA patients. One autopsy study showed that anterior spinal roots were atrophic, and that there was loss of myelinated fibres with abundant glial bundles in a SMA type 3 patient (Kuru et al., 2009). An autopsy study of a SMA type 1 patient showed degeneration of anterior horn neurons and group atrophy of skeletal muscles (Madakshira et al., 2020). A post-mortem study of 5 infants with SMA type 1 showed delayed maturation of acetylcholine receptor subunits and abnormally formed neuromuscular junctions, thereby presenting neuropathological findings in human SMA that are similar to many findings in animal models (Harding et al., 2015). In addition to autopsy studies, a small number of brain magnetic resonance imaging (MRI) studies has been conducted to better inform on the impact of SMA on the brain (as reviewed by Mugisha et al., 2023). The review showed that several brain abnormalities were reported in patients with infantile onset of SMA, including cortical and subcortical brain abnormalities in white matter, thalamus, basal ganglia, and

hippocampus. In patients with later-onset SMA, an increase of grey matter density in motor areas and reduced cerebellar and lobular volume were observed (Mugisha et al., 2023).

#### **1.2.4. Selective vulnerability: an overview**

As described in the previous section, loss of lower motor neurons, denervation of NMJs, and thereby denervation of associated muscles are major hallmarks of SMA in both patients and mouse models of the disease. Although the sequential breakdown of motor neurons is relatively well defined in SMA, it has become apparent that not all motor neurons are uniformly vulnerable. Some motor neurons are lost early during the course of the disease, while other motor neurons stay intact until late stages of the disease. This phenomenon is called selective vulnerability of motor neurons and their associated muscles, and it is a well-documented feature in SMA patients and SMA-like mouse models. Despite numerous reports of the presence of selective vulnerability, the reasons for this phenomenon remain poorly understood.

It has long been recognised that severe SMA patients show selective muscle vulnerability. A profound weakness is present in the core muscles, and the proximal upper and lower limb muscles, with the legs being more affected than the arms and the proximal limb muscles being more affected than the distal limb muscles (Dubowitz, 2009, 1999). A comparative sparing seems to be present in the muscles of the distal upper and lower limbs, hands, feet, diaphragm, and the face (Dubowitz, 2009, 1999). The pattern of muscle weakness in SMA can therefore be described as a symmetrical, predominantly proximal extremity weakness that also affects axial, intercostal, and bulbar muscles (Dubowitz, 2009). A more recent study examined muscle function using the hammer-smith motor function scale and demonstrated that muscle location was a predictor of relative weakness (Wadman et al., 2018). They found that muscle weakness was more profound in proximal limb muscles compared to distal limb muscles, and that proximal leg weakness preceded weakness in the proximal arms (Wadman et al., 2018). In line with finding from Dubowitz, they showed that the triceps, deltoid, iliopsoas and quadriceps are the weakest muscles examined in patients from all types of SMA, and that the

strength of hands and fingers were relatively preserved (Wadman et al., 2018). Further, a study in SMA type 3 patients showed, that the patterns of vulnerability were remarkably uniform over a period of 5-10 years of the disease (Deymeer et al., 2008). They showed that weakness started in the triceps, then biceps and deltoid for upper extremity muscles, and in the lower extremity muscles it started first in the thigh adductors, then iliopsoas, then quadriceps femoris, then hamstrings, thigh abductors, and finally gluteus maximus (Deymeer et al., 2008). Collectively these results show that SMA patients demonstrate a vulnerability pattern of increased vulnerability in the core and proximal leg muscles, followed by the proximal arm muscles with a comparative sparing of distal limb muscles.

Selective vulnerability of muscles and motor neurons is a feature that was also consistently observed in severe and intermediate mouse models of SMA. Previous work on the severe *Smn*<sup>-/-</sup>; *SMN2* mouse model (Monani et al., 2000) investigated head muscles, thoracoabdominal muscles and distal hindlimb muscles (Murray et al., 2008, 2010a; Thomson et al., 2012). Murray et al. investigated cranial muscles in this mouse model and found that some of these muscles are vulnerable while others are resistant at disease end-stage (Murray et al., 2008). A further study also investigated the cranial muscles in this model, in contrast to the study by Murray and colleagues, showing that all but one head muscle at disease end-stage were highly vulnerable (Thomson et al., 2012). They further showed that core and hindlimb muscles presented with different levels of vulnerability, appearing to sit on a spectrum of vulnerability (Thomson et al., 2012).

A comprehensive study in the Taiwanese mouse model (Hsieh-Li et al., 2000) investigated the levels of NMJ loss in 25 axial and appendicular muscles at disease end-stage (Lin et al., 2016). Out of all the investigated muscles throughout the body, only two heads of the flexor digitorum brevis (FDB2 and FDB3) were severely denervated, all other muscles, including the last head of the FDB (FDB4) were either fully resistant or only showed very mild signs of denervation (Lin et al., 2016). Consistent with the findings from Lin et al.,

analysis of the quadratus lumborum (QL) and TA muscles demonstrated that both these muscles only showed mild signs of denervation (Buettner et al., 2021). These findings of a relative lack of neuromuscular pathology in this model were supported by the lack of any significant motor neuron loss in the thoracic and lumbar spinal cord regions in the Taiwanese mouse model (Buettner et al., 2021).

To date, the most characterisations of selectively vulnerable muscles have been performed in the severe SMN $\Delta$ 7 mouse model. A study on the SMN $\Delta$ 7 mouse model (Le et al., 2005) showed that the cranial muscles in this model were not uniformly affected at disease end-stage (Murray et al., 2008). Analysis of muscles of the face, core, abdomen and limbs showed varying levels (25-75%) of pre-synaptic defects in this model (Kariya et al., 2008). Ling et al. performed a comprehensive study of axial and appendicular muscles throughout the body of the SMN $\Delta$ 7 mouse model, thereby investigating 23 muscles at disease end-stage (Ling et al., 2012). Various head, neck, back, thoracic, proximal forelimb, and proximal and distal hindlimb muscles were investigated. They found that the investigated muscles demonstrated a wide range of vulnerability across axial and appendicular muscles, with some being fully resistant and others showing more than 50% synaptic loss. Further, previous work determined that the lumbrical muscles showed a high percentage of synaptic loss (Comley et al., 2016), but these findings are in contrast to a study where they did not find any denervation in the lumbrical muscles at disease end-stage (Ling et al., 2012). The observed selective muscle vulnerability in the SMN $\Delta$ 7 mouse model was further supported by findings of selectively vulnerable motor neurons in the spinal cord. Here, a significant loss of L5 lumbar medial motor neurons (innervating axial muscles) was observed, while lateral motor neurons (innervating distal limb muscles) numbers were not reduced at disease end-stage (Mentis et al., 2011).

Selective muscle vulnerability in the intermediate *Smn*<sup>2B/-</sup> mouse model (Bowerman et al., 2012a) has previously only been investigated in a small number of muscles. Murray et al. investigated cranial muscles and abdominal muscles at disease end-stage, showing that cranial muscles are resistant to

denervation while abdominal muscles are vulnerable and display high levels of synaptic loss in the *Smn*<sup>2B/-</sup> mouse model (Murray et al., 2015, 2013). That is in line with a study that also demonstrates that the abdominal TVA muscle is a vulnerable muscle with high levels of NMJ loss in this mouse model (Courtney et al., 2019). Previous work further investigated the QL and TA muscles, showing that the QL shows high levels of synaptic loss, while the TA only shows mild denervation at disease end-stage P22 (Buettner et al., 2021). Selective vulnerability in this model was further supported by findings showing that motor neurons from thoracic and upper lumbar spinal cord regions are lost in this model (Bowerman et al., 2012a; Buettner et al., 2021; Courtney et al., 2019), while motor neuron numbers remain intact in lower lumbar spinal cord regions at disease end-stage (Buettner et al., 2021; Carlini et al., 2022).

This selective motor neuron and muscle vulnerability observed in SMA patients and mouse models of the disease can be utilised as a tool to further our understanding of the underlying cellular and molecular mechanisms of the disease. This phenomenon of selective vulnerability also provides an opportunity to investigate mechanisms that drive motor neuron protection, which can help reveal modifiers of the disease, thereby providing a platform for the development of future combinational therapeutics which will target the most vulnerable motor units. It is critical to further profile this selective vulnerability in experimental models to gain a better understanding of the pathomechanisms of SMA.

### **1.3. Important disease modifiers of SMA**

#### **1.3.1. Modifiers of SMA associated with well-described disease mechanisms**

The genetics as well as the neuropathological hallmarks of SMA, including motor neuron and NMJ vulnerability and defects, have been well-described over the past two decades. Despite this, the exact mechanistic connections between molecular genetics and resulting neuromuscular defects are yet to be further discerned. However, various molecular mechanisms that underlie SMA could be described through the identification of causative or modifier genes from animal models of the disease. A number of cellular pathways has been

highlighted that could contribute to synaptic and neuromuscular dysfunctions in SMA, including axonal transport and cytoskeletal integrity, mitochondrial function, RNA processing, and growth factor signalling (Murray et al., 2010b). The identification of modifier genes associated with these pathways has been possible by utilising the selective vulnerability of motor neurons in animal models of the disease. As previously discussed, mouse models of SMA show distinct selective vulnerability of certain motor neuron groups and associated muscles, with some being resistant and some being highly vulnerable at disease end-stage. Utilising these differentially vulnerable motor neuron populations for next generation sequencing approaches allows the comparison and profiling of these distinct populations, thereby facilitating the identification of potential modifier genes of the disease.

A molecular mechanism that has been shown to be disrupted in SMA is axonal transport and the integrity and architecture of the cytoskeleton. As previously discussed, neurofilaments are an integral part of the cytoskeleton and there are a number of defects seen in axonal transport functions and at the NMJ that are associated with SMA (Cifuentes-Diaz et al., 2002; Kariya et al., 2008; Kong et al., 2009; Murray et al., 2008). However, the cytoskeleton is not just composed of neurofilaments, but it also incorporates key components such as microtubules and actin protein. All these cytoskeletal components hold fundamental roles in the neuronal architecture and function. A study by Kline and colleagues performed a comparison of four independent screens (three ALS screens and one SMA screen) of differentially vulnerable motor neuron populations from neurologically normal mice, rats and humans, three of which were performed in ALS and one on a mouse model of SMA (Kline et al., 2017). This multi-model study revealed that Stathmin (*STMN1*) was significantly downregulated in vulnerable motor neurons compared to resistant motor neurons, thereby suggesting that its expression correlates with resistance to pathology as a potential modifier of SMA. *STMN1* is a ubiquitously expressed microtubule binding phosphoprotein that plays an important role in microtubule dynamics and neuronal cytoskeleton dynamics, including mitosis, motility, process formation and intracellular transport (Rubin and Atweh, 2004). AAV9-mediated treatment of the intermediate *Smn*<sup>2B/-</sup> mouse model of SMA with

*Stmn1* revealed that it indeed functioned as modifier of SMA pathology (Villalón et al., 2019). *Stmn1*-treated SMA mice showed a significant increase in lifespan and motor function, a decrease in synaptic loss in vulnerable abdominal muscles, and an improvement in the filamentous nature of the tubulin networks that surround neuronal cell bodies in comparison to their untreated controls (Villalón et al., 2019). These findings further confirm the link between neuromuscular pathology in SMA and disruptions in molecular mechanisms associated with cytoskeleton dynamics, suggesting that improving cytoskeletal integrity and dynamics can improve the observed SMA phenotype.

Another molecular mechanism that is closely linked to SMA pathology, are mitochondrial dysfunctions and defects in mitochondrial bioenergetics. The role of mitochondria is mainly associated with energy and metabolic pathways, in turn leading to a suboptimal energy production and supply for cells when disrupted. As neuronal and muscle cells are some of the most energy dependent cells of the body, a disruption in mitochondrial function and bioenergetics can be especially troubling for these cell types. Countless publications have described mitochondrial dysfunctions in diverse models of SMA, including defects in oxidative phosphorylation and mitochondrial respiration, reactive oxygen species and oxidative stress, mitochondrial metabolism, mitochondrial trafficking, apoptosis, mitochondrial dynamics, and mitochondrial biogenesis and mitophagy (as reviewed by James et al., 2021). Amidst the numerous publications of mitochondrial dysfunctions in SMA, work by Boyd and colleagues utilised differentially vulnerability motor neuron populations from neurologically normal mice to conduct a microarray study and identified a list of potential modifier genes of SMA (Boyd et al., 2017). They identified an increased expression of phosphoglycerate kinase 1 (*Pgk1*) in resistant motor neurons, a key glycolytic enzyme that generated the first molecule of ATP in the glycolytic process. The main function of mitochondria is to generate ATP from oxidative phosphorylation, with efficient ATP production being of particular importance for tissues with high energy demands, including motor neurons. In this study they investigated the neuroprotective effects of *Pgk1* in a zebrafish model of SMA, showing that the

overexpression of *Pgk1* led to a significant rescue effect of the axonal outgrowth phenotype, including a decrease of axonal defects and an increase in normal motor neurons in this zebrafish model (Boyd et al., 2017). These findings strengthen the understanding of the role of ATP-dependent pathways in regulating selective vulnerability of motor neurons in SMA, and further confirm the link between mitochondrial dysfunctions and neuromuscular pathology in SMA.

The perhaps most extensively described molecular mechanism of SMA is the role of SMN protein in RNA processing with its widespread splicing defects that are causative of SMA pathology (see section 1.1.1. for detailed description). It has long been established that SMN is an RNA binding protein, with work generally focused on its role on pre-mRNA splicing, mRNA transport, and local RNA translation (as reviewed by Chaytow et al., 2018). These diverse roles of SMN protein in the pathology of SMA have further been confirmed by findings from Murray and colleagues in an intermediate mouse model of SMA. This study utilised differentially vulnerable motor neuron populations from pre-symptomatic *Smn*<sup>2B/-</sup> mice and healthy control littermates to conduct a RNA-seq screen that identified a list of potential modifiers of the disease (Murray et al., 2015). They found a down regulation of factors involved in rRNA binding and a selective up regulation of cell death pathways in selectively vulnerable motor neurons (Murray et al., 2015). A decrease of factors involved in the positive regulation of DNA repair was observed in vulnerable motor neurons, thereby suggesting that the response to DNA damage is decreased in selectively vulnerable motor neurons in this mouse model of SMA (Murray et al., 2015).

### **1.3.2. Other important modifiers of SMA**

By utilising selective vulnerability and the associated differentially vulnerable motor neuron populations, a number of disease modifiers have been identified that are closely linked to mechanistic and causative pathways of SMA. However, this approach has also identified some disease modifiers that are not immediately associated with one of the most well-known molecular mechanisms of SMA. These other valuable modifiers have the potential to

inform on yet unknown links between neuropathology of the disease and mechanistic pathways of SMA.

The same comparative multi-model study of ALS and SMA screens from neurologically normal mice, rats and humans that identified *STMN1* as disease modifier, also identified alpha-synuclein (*SNCA*) as a potential modifier of SMA (Kline et al., 2017). The study revealed that *SNCA* was consistently downregulated in vulnerable motor neurons compared to resistant motor neurons across all four screens. Mutations in *SNCA* bear a strong association with aggregate formation, which leads to the degeneration of nigrostriatal neurons, causing a common disorder of the basal ganglia. *SNCA* is further associated with the release of neurotransmitters. AAV9-mediated treatment of the intermediate *Smn*<sup>2B/-</sup> mouse model of SMA with *SNCA* revealed that *SNCA* expression increased the median lifespan and ameliorated the disease phenotype in treated SMA mice compared to untreated controls (Kline et al., 2017). They further observed an amelioration of the NMJ phenotype, showing that there was a significant increase in the percentage of fully occupied endplates in treated SMA mice compared to untreated controls, thereby indicating a decrease in denervation and motor neuron pathology (Kline et al., 2017). *SNCA* was shown to work as a modifier of the disease, ameliorating the disease phenotype.

A recent study utilised a variety of vulnerable and resistant motor neuron populations from SMNΔ7 and healthy control littermates in a longitudinal transcriptomics analysis (Nichterwitz et al., 2020). They found that growth differentiation factor 15 (*GDF15*) was highly up-regulated in resistant motor neurons from CN III/ CN IV from SMA mice compared to vulnerable motor neurons (Nichterwitz et al., 2020). *GDF15* is a cytokine that is involved in the stress response program of cells after cellular injury and the promoting of neuronal survival. They hypothesised that *GDF15* could be a potential disease modifier as adaptation mechanisms identified in resistant motor neurons could be used to reduce susceptibility in vulnerable motor neurons. The study investigated the potential neuroprotective effect of *GDF15* on vulnerable spinal motor neurons generated from human spinal motor neurons from induced

pluripotent stem cells (iPSCs). They demonstrated that adding *GDF15* to the vulnerable spinal motor neurons in culture, led to a significant improvement of their survival in a dose-dependent manner, thereby validating *GDF15* as a modifier of motor neuron vulnerability in SMA (Nichterwitz et al., 2020).

Recent work conducted a comparative study of two microarray screens on resistant and vulnerable motor neuron populations in ALS mice and rats (Nizzardo et al., 2020). They found that Synaptotagmin 13 (*SYT13*) is enriched in resistant motor neurons compared to vulnerable motor neurons. *SYT13* is a vesicular trafficking protein important for synapsis and vesicle metabolism. They hypothesised that *SYT13* could be a modifier of the disease as it is enriched in resistant motor neurons (Nizzardo et al., 2020). Nizzardo and colleagues tested the neuroprotective properties of *SYT13* in iPSCs generated from SMA patient motor neurons and in the *SMNΔ7* mouse model of SMA. They demonstrated that the overexpression of *SYT13* significantly increased the survival of SMA patient motor neurons, thereby protecting vulnerable motor neurons from degeneration in SMA (Nizzardo et al., 2020). AAV9-mediated *Syn13* treatment of mice from the *SMNΔ7* mouse model, showed a prolonged median lifespan, and an improvement in motor performance in treated SMA mice compared to untreated controls (Nizzardo et al., 2020). They further demonstrated that *Syn13* treatment preserved motor neurons, increased motor neuron soma size and reduced levels of NMJ denervation in treated compared to untreated SMA mice (Nizzardo et al., 2020). *SYN13* was thereby shown to be a modifier for phenotypic and neuromuscular pathology in SMA patient motor neurons and an animal model of the disease.

Overall, it is evident that the utilisation of differentially vulnerable motor neuron populations for spatial transcriptomics has been able to identify important modifiers of disease. Selective vulnerability thereby represents a valuable tool to investigate potential genetic modifiers of the disease and how those can further inform on molecular mechanisms of SMA.

#### 1.4. Aims of thesis

The work performed within this thesis aims to investigate modifiers that have the potential to regulate selective vulnerability in mouse models of SMA. Despite proven selective vulnerability of motor neurons and associated muscles in both patients and mouse models of SMA, the mechanisms underlying and regulating this selective vulnerability remain largely unknown. Identifying and investigating modifiers that can confer motor neuron protection becomes increasingly important in our pursuit of potential targets that can be utilised for new therapeutic strategies.

### Chapter 3

#### **Aims:**

1. Perform a thorough body wide characterisation of selectively vulnerable muscles and associated motor neurons in the *Smn*<sup>2B/-</sup> mouse model to identify the selective vulnerability pattern present in this mouse model of SMA
2. Gather, analyse and compare the patterns of selective vulnerability in the four most commonly utilised mouse model of SMA

#### **Rational:**

Selective vulnerability of motor neurons and associated muscles is a widely documented phenomenon in SMA patients and mouse models of the disease. However, the underlying mechanisms of selective vulnerability are still unclear. While a variety of selectively vulnerable muscles has been characterised in severe mouse models of SMA (Buettner et al., 2021; Comley et al., 2016; Lin et al., 2016; Ling et al., 2012; Murray et al., 2010a, 2008; Thomson et al., 2012), a thorough characterisation of muscles from an intermediate mouse model of SMA is currently lacking. Although there have been various publications investigating NMJ pathology and synaptic loss in the four most commonly utilised mouse models of SMA, there has not yet been a comprehensive comparison of the resulting patterns of selective vulnerability in these models. A direct comparison of the similarities and differences of selective vulnerability patterns between models, can facilitate the

understanding and identification of conserved mechanisms and temporal series of degenerative and developmental events that underlie motor neuron and NMJ pathology in mouse models of SMA.

**Hypotheses:**

1. Selective vulnerability of muscles is a feature present in the *Smn*<sup>2B/-</sup> mouse model of SMA
2. Motor neuron cell body loss will correlate with NMJ pathology in the *Smn*<sup>2B/-</sup> mouse model
3. The four most commonly utilised mouse models of SMA will show similar selective vulnerability patterns

**Chapter 4**

**Aims:**

1. Perform a cross-model transcriptional analysis, comparing differentially vulnerable CN VII motor neuron populations from two mouse models of SMA (vulnerable population in the SMN $\Delta$ 7 and resistant population in the *Smn*<sup>2B/-</sup> mouse model) to identify groups of transcripts that may confer motor neuron protection
2. Perform a cross-model transcriptional analysis to identify a refined list of potential neuroprotective modifiers in wildtype motor neurons, choosing populations which are known to be vulnerable in SMA mouse models
3. Investigate the neuroprotective effects of PACAP on phenotype, motor performance and neuropathology in the *Smn*<sup>2B/-</sup> mouse model of SMA

**Rational:**

Transcriptional profiling of differentially vulnerable motor neuron populations from different disease models has previously been utilised in ALS and SMA to identify modifiers of disease. It has thereby been shown that selective vulnerability provides an opportunity to investigate mechanisms that can drive motor neuron protection in SMA, revealing potentially interesting modifiers of

disease. Although studies identified important modifiers of disease in ALS (Brockington et al., 2013; Hedlund et al., 2010; Kaplan et al., 2014; Nizzardo et al., 2020) and SMA (Boyd et al., 2017; Murray et al., 2015; Nichterwitz et al., 2020; Nizzardo et al., 2020) by performing transcriptional analysis in a single disease model, previous work showed that multi-model comparisons can further refine lists of potential neuroprotective modifiers (Kline et al., 2017). Performing a cross-model comparison of selectively vulnerable motor neurons from two SMA mouse models, can drastically increase the likelihood of the effective identification of modifiers that confer motor neuron protection in SMA. Results from Chapter 3 of this thesis demonstrated that muscles innervated by the facial nucleus (CN VII) were highly vulnerable in the SMN $\Delta$ 7 mouse model, while all muscles innervated by the CN VII in the *Smn*<sup>2B/-</sup> mouse model were shown to be resistant to degeneration. These vast differences in the selective vulnerability of the same motor neuron population between the SMN $\Delta$ 7 and *Smn*<sup>2B/-</sup> mouse models, makes these differentially vulnerable motor neuron populations great candidates for a transcriptional analysis. The observed sparing of the CN VII motor neurons in the intermediate *Smn*<sup>2B/-</sup> mouse model of SMA, led me to hypothesise that transcripts within this motor neuron population must confer motor neuron protection. The in this thesis identified *Adcyap1* is a transcript that encodes PACAP, a neuropeptide that has been shown to have neuroprotective properties in an array of neurodegenerative disorders. Investigating whether an increase in PACAP levels can improve phenotype and motor performance, and whether it can delay synaptic loss *in vivo* will provide a better understanding of the neuroprotective properties of PACAP in SMA.

### **Hypotheses:**

1. Cross-model transcriptional analysis of differentially vulnerable motor neurons from the CN VII will identify groups of transcripts that may confer motor neuron protection
2. Cross-model transcriptional analysis of differentially vulnerable motor neurons from healthy animals will yield a refined list of potential neuroprotective modifiers by identifying consistent changes between models

3. Increase of PACAP will improve phenotype and motor performance in a mouse model of SMA
4. Increase of PACAP will delay synaptic loss in a mouse model of SMA

## **Chapter 5**

### **Aims:**

1. Optimise, expand and couple existing LCM-sequencing and single cell RNA-sequencing protocols to effectively isolate single motor neurons and produce cDNA libraries of these single motor neurons from the CN VII of the SMN $\Delta$ 7 mouse model of SMA
2. Demonstrate that the prepared cDNA libraries yielded good quality sequencing results and that they can be utilised for in-depth sequencing

### **Rational:**

Bulk RNA-sequencing from differentially vulnerable motor neurons has previously been utilised to effectively identify modifiers of disease in SMA mouse models (Kline et al., 2017; Murray et al., 2015; Nichterwitz et al., 2020). While bulk RNA-sequencing is a useful tool in the identification of potential modifiers, it can only outline the collective mean expression levels of the entirety of all cell types represented within a specific tissue sample. Single cell RNA-sequencing on the other hand can be utilised as a more targeted approach to investigate the transcriptional differences between cell subpopulations within a highly heterogenic tissue sample. The optimisation and extension of existing single cell RNA-sequencing (Schweingruber et al., 2023) protocols, coupled with RNA-sequencing that uses LCM for tissue isolation (LCM-sequencing) protocols (Nichterwitz et al., 2018, 2016), will allow the investigation of specific cellular signature profiles from different motor neuron subpopulations. In this thesis it was demonstrated that the facial nucleus (CN VII) is a highly heterogeneous cell population in a severe mouse model of SMA. Performing optimised single cell LCM-sequencing on the CN VII of this mouse model facilitates the investigation of specific cellular signature profiles from different motor neuron subpopulations.

**Hypotheses:**

1. The CN VII of the SMN $\Delta$ 7 mouse model of SMA shows a high heterogeneity in motor neuron subpopulations, providing an interesting basis for investigation of specific cellular signature profiles from motor neuron subpopulations
2. Optimised single cell LCM-sequencing protocol will allow effective isolation of single motor neurons from the CN VII in post-mortem tissue from a mouse model of SMA
3. Optimised single cell LCM-sequencing protocol will allow effective preparation of cDNA libraries from single motor neurons
4. cDNA libraries from single motor neurons will yield good quality sequencing results
5. Optimised single cell LCM-sequencing protocol will facilitate the identification of specific cellular signature profiles of motor neuron subpopulations

## Chapter 2

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### Materials and Methods

#### 2.1. Ethics statement

All animal procedures were performed in accordance with UK Home Office guidelines and institutional guidelines from the University of Edinburgh. In accordance with the local Home Office guidelines and the Animals (Scientific Procedures) Act 1986, the University of Edinburgh holds an Establishment Licence (PEL), Lyndsay Murray holds a Project Licence (PPL), and I hold a Personal Licence (PIL) that allows the work with rodents. All regulated procedures on animals underwent local ethical review and approval by the University of Edinburgh through an experimental request form (ERF), to confirm that the proposed procedures are covered by the project licence, that the PIL holder has sufficient training in the procedure, that the requested recourses are available, and that the named veterinary surgeon and the named training and competency officer do not have any concerns in relation to the implementation of the 3Rs (according to the UK-based scientific organisation 'National Centre for the Replacement Refinement & Reduction or Animals in Research').

#### 2.2. Mouse maintenance and phenotypic analysis

##### 2.2.1. *Smn*<sup>2B/-</sup> mice – an intermediate mouse model of SMA

Both, *Smn*<sup>2B/2B</sup> mice and *Smn*<sup>+/-</sup> mice were maintained under pathogen-free conditions, group housed in individually ventilated cages, with *ad libitum* access to food and water, on a 12:12 hours light/dark cycle within animal facilities of the University of Edinburgh. To obtain *Smn*<sup>2B/+</sup> control mice and *Smn*<sup>2B/-</sup> experimental mice, *Smn*<sup>2B/2B</sup> mice (bred on a C57Bl/6J [BL6] congenic background) were interbred with *Smn*<sup>+/-</sup> mice (Jackson Laboratories strain formerly 010921, now 006214, FVB.129P2-*Smn*<sup>1<sup>tm1Msd</sup></sup>/J congenic background; To generate these mice, the target mutant allele was electroporated into 129P2/OlaHsd-derived E14Tg2a-IV embryonic stem (ES) cells and ES cells were injected into C57BL/6 blastocysts to obtain chimeric

animals. Chimeric animals were then crossed to C57BL/6 and then crossed to a strain carrying the Tg(SMN2)89Ahmb transgene. Those double mutants were then backcrossed to FVB/N for at least 5 generations and were then bred to the FVB background to select for the targeted mutation only.). The *Smn*<sup>2B/-</sup> mouse is an intermediate mouse model of the childhood motor neuron disease. In our hands, animals display motor symptom onset around P8, and a lifespan of around 18 days. As there are no known differences in phenotype between male and female animals, animals of either sex were sacrificed at disease end stage, between P16 and P18, depending on the experimental design. If abdominal muscle preparations were needed, animals were sacrificed by inhalation overdose of anaesthetic (isoflourane) and death was confirmed by severing of the carotid artery, whilst those where no abdominal preparations were needed, were sacrificed by intraperitoneal (IP) injection overdose of anaesthetic (pentobarbital) and death was confirmed by severing of the carotid artery. Mice sacrificed for other work were sacrificed by rising CO<sub>2</sub> levels and death was confirmed by severing the carotid artery.

### **2.2.2. Survival, weight and motor testing in *Smn*<sup>2B/-</sup> mice**

To test the effects of PACAP treatment (as presented in Chapter 4), mice were closely monitored for signs of weight gain and improvement of motor skills. IP PACAP and AAV9-PACAP injected animals underwent daily monitoring by researchers and by animal technicians working in the animal facility at the University of Edinburgh, to assess their general wellbeing and detect any signs of distress (as described by Langford et al., 2010). No official distress scoring was recorded. For treatment via intraperitoneal (IP) injection of the PACAP27 or PACAP38 protein, mice were weight daily from P5 onwards, and monitored for signs of prolonged survival and weight gain. Motor testing of mice was performed once at the end of their lifespan before sacrificing them at disease end-stage. The motor test performed is termed the 'Time to Turn Around Test' (TTT). Mice were placed with their head downwards on a gridded cage top that was placed at a 45° angle. The time it took the mice to turn around 180° on the grid and face upwards was measured. The test was stopped once the animal turned successfully or after 30 seconds of an unsuccessful attempt (Figure 2.1). To report the *in vivo* experiments conducted on the *Smn*<sup>2B/-</sup> and control

animals in this thesis, as many ARRIVE guidelines as possible were followed, including guidelines on study design, statistical methods, experimental animals, experimental procedures and results (Sert et al., 2020).

### **2.2.3. SMN $\Delta$ 7 mice – a severe mouse model of SMA**

The FVB/NCrl *Smn*<sup>-/-</sup>;*SMN2*<sup>2copy</sup>;*SMN $\Delta$ 7*<sup>2copy</sup> (herein referred to as SMN $\Delta$ 7) mice were maintained under pathogen-free conditions, group housed in individually ventilated cages, with *ad libitum* access to food and water, on a 12:12 hours light/dark cycle within animal facilities of the University of Edinburgh. *Smn*<sup>+/-</sup>;*SMN2*<sup>2copy</sup>;*SMN $\Delta$ 7*<sup>2copy</sup> (Jackson strain 005025, FVB.Cg-*Grm7*<sup>Tg(SMN2)89Ahmb</sup>*Smn1*<sup>tm1Msd</sup>Tg(SMN2\*delta7)4299Ahmb/J congenic background) were interbred to produce *Smn*<sup>-/-</sup>;*SMN2*<sup>2copy</sup>;*SMN $\Delta$ 7*<sup>2copy</sup> (aka SMN $\Delta$ 7). The SMN $\Delta$ 7 mouse is a severe mouse model of the childhood motor neuron disease SMA. In our hands, symptom onset is around P4-P5 and this model has a lifespan of around 10-12 days. Female and male animals were sacrificed at various stages of the disease. Animals taken at P2 were sacrificed by decapitation, whilst animals taken at P5, P9, P10 and P12 were sacrificed by decapitation under terminal anaesthesia.

### **2.2.4. Survival, weight and motor testing in SMN $\Delta$ 7 mice**

Because of the observed variation in lifespan of 10-12 days in these animals, it was important to determine the appropriate end of lifespan in these mice to collect tissue for LCM (as presented in Chapter 5). Animals needed to be sacrificed immediately, if their motor abilities and general health deteriorated enough for them to be unable to feed independently any longer. To assess the appropriate lifespan, animals were closely monitored for weight loss and deficits in motor skills that would exacerbate their survival. Animals underwent daily monitoring by researchers and by animal technicians working in the animal facility at the University of Edinburgh, to assess their general wellbeing and detect any signs of distress (as described by Langford et al., 2010). No official distress scoring was recorded. Mice were weighed, and their motor skills tested daily with the 'Time to Right Test' (TTR) from P8 onwards. For the TTR, mice were placed on their back with none of their four paws touching the ground. Time was taken that it took the mice to right themselves and turn back

onto all four paws (Figure 2.1). The test was stopped once the animal turned successfully or after 30 seconds of an unsuccessful attempt. As SMN $\Delta$ 7 pups were often unable to successfully perform this test from P10 onwards, additional marker for their motor abilities and general health were considered during observation. Mice were immediately sacrificed if their weight dropped by more than 20% within 48h, if the animals were no longer able to stand on all 4 paws for longer than 5 seconds, and if they did no longer try to right themselves in the TTR test while seeming lethargic, fatigued and unresponsive to stimuli. To report the *in vivo* experiments conducted on the SMN $\Delta$ 7 and control animals in this thesis, as many ARRIVE guidelines as possible were followed, including guidelines on study design, statistical methods, experimental animals, experimental procedures and results (Sert et al., 2020).

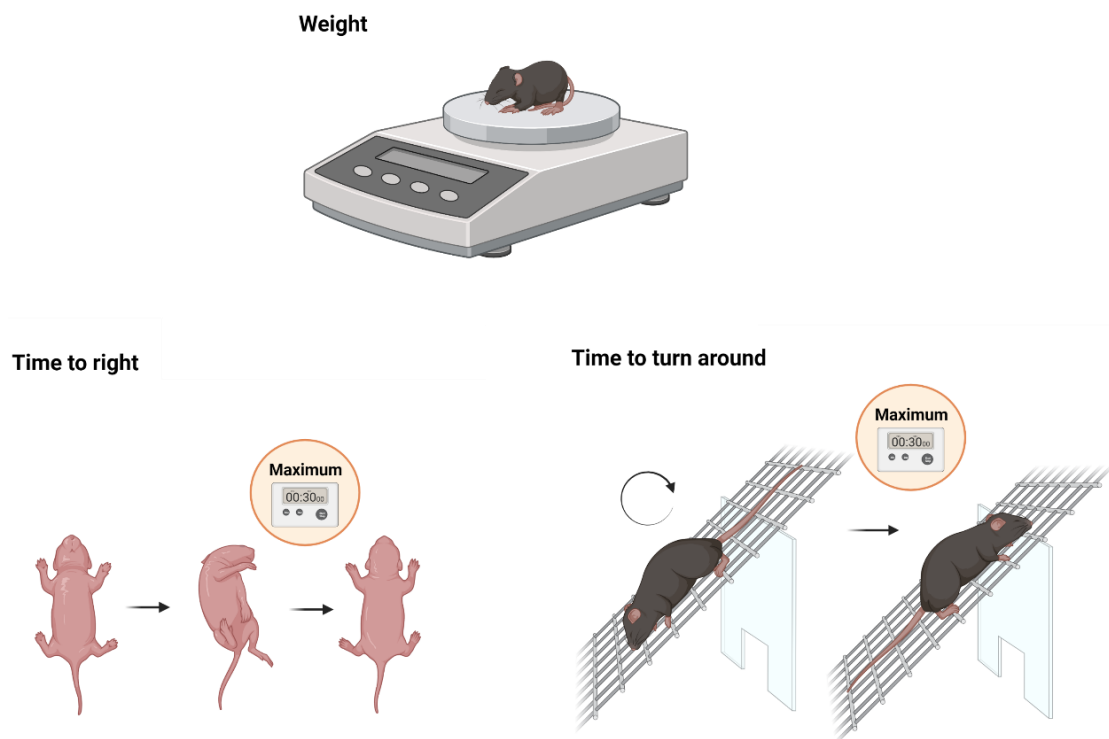


**Figure 2.1: Phenotypic analysis of *Smn*<sup>2B/-</sup> and SMN $\Delta$ 7 mice through weight and motor tests.** The Figure shows how the weighing and motor tests of *Smn*<sup>2B/-</sup> and SMN $\Delta$ 7 mice and their control animals were performed. Shown are the time to right test used in the SMN $\Delta$ 7 mouse model and the time to turn test used in the *Smn*<sup>2B/-</sup> mouse model. Note that all motor tests had a maximum test duration of 30 seconds before they were terminated.

## 2.3. Genotyping

### 2.3.1. DNA extraction

DNA was extracted from tail tips or ear notches by digesting the tissue in 500µL of tail tip lysis buffer (TTLB; 0.5M EDTA, 1M Tris- HCl, 1M NaCl, 10% SDS in ddH<sub>2</sub>O) and 2.5µL of Proteinase K (1mg/mL) for 24 hours at 55°C. DNA was precipitated in isopropanol and washed twice with 70% ethanol. DNA was dried, and then re-suspended in 200µL ddH<sub>2</sub>O for tail tips or in 50µL ddH<sub>2</sub>O for ear notches. DNA concentration could be measured with a nanodrop if required, with an expected concentration of approximately 200ng/µL. DNA was stored at -20°C.

In some cases, an alternative and quicker method was applied if genotyping was required promptly. This method allowed to finish DNA extraction within a couple of hours. DNA was extracted from ear notches or tail tips by digestion in 300µL or 600µL of 50mM NaOH respectively. The tissue samples were subsequently heated to 95°C for 10 minutes. 1M Tris-HCl (pH 8.0) was added to the DNA and then centrifuged for 6 minutes. The resulting DNA supernatant was taken off and transferred to a fresh tube. DNA was stored at -20°C.

### 2.3.2. PCR Protocols

For identification of appropriate genotypes in different SMA mouse models, appropriate PCR protocols were utilised. The *Smn*<sup>+/-</sup> protocol was used in genotyping of both the *Smn*<sup>2B/-</sup> and *SMN*Δ7 mouse model. It was used to determine whether the murine *Smn* allele was functional or non-functional (null) in mice. PCR was preformed using an Invitrogen PCR kit with a common forward primer and two reverse primers. One reverse primer binds to the wild-type genetic sequence, the other binds to the inserted cassette that creates the null allele (Table 2.1). 10µL of master mix (Table 2.2) were added to 2µL of DNA and set to a pre-programmed temperature cycle (Table 2.3).

Gel electrophoresis, using a 1.5% agarose gel that contained 0.01% SYBR Safe, separated the DNA strands created from this PCR protocol. An upper 800bp band identified the wild-type *Smn* allele and a lower 500bp band identified the null *Smn* allele. A null (non-functioning) *Smn* allele results in no

*Smn* product from this allele and can be used to distinguish between mice with SMA phenotype and control mice with no SMA phenotype.

In the *Smn*<sup>2B/-</sup> mouse model, control *Smn*<sup>2B/+</sup> mice can be identified by having a functioning *Smn* allele (800bp band only), whereas *Smn*<sup>2B/-</sup> mice present with a null *Smn* allele (500bp band) in addition to the functioning wildtype *Smn* allele (800bp band). In the SMNΔ7 mouse model, control *Smn*<sup>+/+</sup> and *Smn*<sup>+/-</sup> can be identified by having a functioning *Smn* allele (800bp band only, or 800bp and 500bp band), whereas SMNΔ7 (*Smn*<sup>-/-</sup>) mice present with a null *Smn* allele (500bp band only).

**Table 2.1: PCR Primer Sequences for Genotyping**

| Protocol                  | Primer Sequence                                                                                                                                               |
|---------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Smn</i> <sup>+/-</sup> | Forward Common: 5'- CTC CGG ATA TTG GGA TTG -3'<br>Reverse Wild-type: 5'- TTT CTT CTG GCT GTG CCT TT -3'<br>Reverse Mutant: 5'- GGT AAC GCC AGG GTT TTC C -3' |
| SMNΔ7                     | Forward Common: 5'- CTC CGG ATA TTG GGA TTG -3'<br>Reverse Wild-type: 5'- TTT CTT CTG GCT GTG CCT TT -3'<br>Reverse Mutant: 5'- GGT AAC GCC AGG GTT TTC C -3' |

**Table 2.2: *Smn*<sup>+/-</sup> and SMNΔ7 PCR Protocol Master Mix Components**

| Reagent                  | Volume (μL) per sample |
|--------------------------|------------------------|
| Water                    | 4.85                   |
| Buffer                   | 0.97                   |
| MgCl <sub>2</sub>        | 0.48                   |
| dNTPs (Invitrogen)       | 0.24                   |
| Forward Common Primer    | 1.60                   |
| Wild-type Reverse Primer | 1.20                   |
| Mutant Reverse Primer    | 0.60                   |
| Taq                      | 0.05                   |

**Table 2.3: *Smn*<sup>+/-</sup> and SMN $\Delta$ 7 PCR Protocol Temperature Cycle**

| Step Number              | Temperature (°C) | Time          |
|--------------------------|------------------|---------------|
| 1.                       | 94°C             | 3 minutes     |
| 2.                       | 94°C             | 30 seconds    |
| 3.                       | 58°C             | 1 minute      |
| 4.                       | 72°C             | 1 minute      |
| Repeat steps 2-4 for 35x |                  |               |
| 5.                       | 72°C             | 2 minutes     |
| 6.                       | 4°C              | Infinite hold |

## 2.4. PACAP dosing

### 2.4.1. IP injection dosing regimen

*Smn*<sup>2B/-</sup> pups and control littermates were injected via intraperitoneal injections with FITC-conjugated PACAP27 or PACAP38 (kindly provided by David Chatenet from the University of Quebec). The treatment was administered daily from P5 onwards until sacrificed. Animals were sacrificed at disease end-stage, 2 hours after the last dose of PACAP or saline was administered, for the investigation of neuroprotective effects of PACAP. Half of the animals in each litter received a dosage of 182.49mg/kg PACAP27 diluted in saline or a dosage of 251.8mg/kg PACAP38 diluted in saline, the other half of the litters received 2μL/g saline as a vehicle control. IP injections were administered by technical staff of the animal facilities at the University of Edinburgh. Pups were closely monitored after treatment to ensure they maintain the expected condition and that maternal care and feeding was resumed. All animals were weighed daily to calculate injection volumes and for monitoring purposes. If more than 20% of their body weight was lost over the course of 48h, the animal was sacrificed.

### 2.4.2. AAV9 ICV injection regimen

*Smn*<sup>2B/-</sup> pups and control littermates were injected via ICV injections with a AAV9-PACAP-GFP viral vector. A single treatment of AAV9-PACAP-GFP was administered at P1 (for low dose AAV9-PACAP) or at P2 or P3 (for high dose AAV9-PACAP) with help from Laura Comley and Inga Partlova. Animals in

each litter either received 3  $\mu\text{L}$  of a low dose AAV9-PACAP-GFP viral vector diluted in saline to a concentration of  $3.33 \times 10^{10} \text{VG/mL}$  or 3  $\mu\text{L}$  of saline as control group, or they received 5  $\mu\text{L}$  of the undiluted high dose of  $8.07 \times 10^{12} \text{VG/mL}$  AAV9-PACAP-GFP viral vector or 5  $\mu\text{L}$  of saline as respective control group, or they remained untreated. Animals were sacrificed at P18 (disease end-stage) to investigate the neuroprotective effects of PACAP in various tissues. Pups were closely monitored after treatment to ensure they maintain the expected condition and that maternal care and feeding was resumed. If more than 20% of their body weight was lost over the course of 48h, the animals were sacrificed.

## **2.5. Immunohistochemistry, quantification, and statistical analysis**

### **2.5.1. Immunohistochemistry for NMJ analysis on muscles**

In preparation for NMJ labelling, muscles were immediately gross dissected from recently sacrificed mice. Here, depending on the desired muscles, entire body regions were removed from the mice for subsequent fixation and microdissection. The gross dissected body regions included the tissue between the ears for cranial muscles, the head and neck for facial and neck muscles, the ribcage for thoracic muscles, the abdominal wall for abdominal muscles, the forelimb for proximal and distal forelimb muscles, and the hindlimb for proximal and distal leg muscles as well as foot muscles. All dissected body regions were fixed in 4% paraformaldehyde (PFA, Electron Microscopy Science) in 1x phosphate buffered saline (PBS) for 15 minutes. Tissues were then washed in 1xPBS. Subsequently, all muscles of interest were microdissected post fixation (Figure 2.2). Each muscle was carefully detached from surrounding bones and tissues of the associated body region, ensuring that muscles were not damaged during fine dissection. Muscles were cleaned from excess fascia and connective tissue and washed in 1xPBS. The fixed and microdissected muscles were permeabilised in 2% Triton X-100 (Sigma, PTB) in 1xPBS for 30 minutes using a multiwell plate on a rocking platform at room temperature. Muscles were blocked in blocking solution (4% bovine serum albumin [BSA]/1% Triton X-100 in 1xPBS) for 30 minutes on a rocking platform at room temperature followed by overnight incubation in primary antibodies against synaptic vesicle protein 2 (SV2) and neurofilament

(NF, 2H3) (SV2, 1:100; NF; SH3, 1:50, both from Developmental Studies Hybridoma Bank) in blocking solution, left to incubate on a rocking platform at 4°C for 24 hours. After the overnight incubation, tetramethylrhodamine-conjugated  $\alpha$ -bungarotoxin (BTX, 1:250, Thermo Fisher) in 1xPBS was applied for 60 minutes at room temperature. Muscles were then washed three times for 5 minutes with 1xPBS. Muscles were incubated in secondary antibody (rabbit anti-mouse, 1:250, Alexa Fluor 488, Jackson ImmunoResearch Laboratories) in 1xPBS for 3-4 hours on a rocking platform in the dark at room temperature and then washed three times with 1xPBS. Muscles were whole-mounted on glass slides (Thermo Fisher) using Mowiol® (Calbiochem) and cover-slipped. Slides were stored at 4°C in the dark.

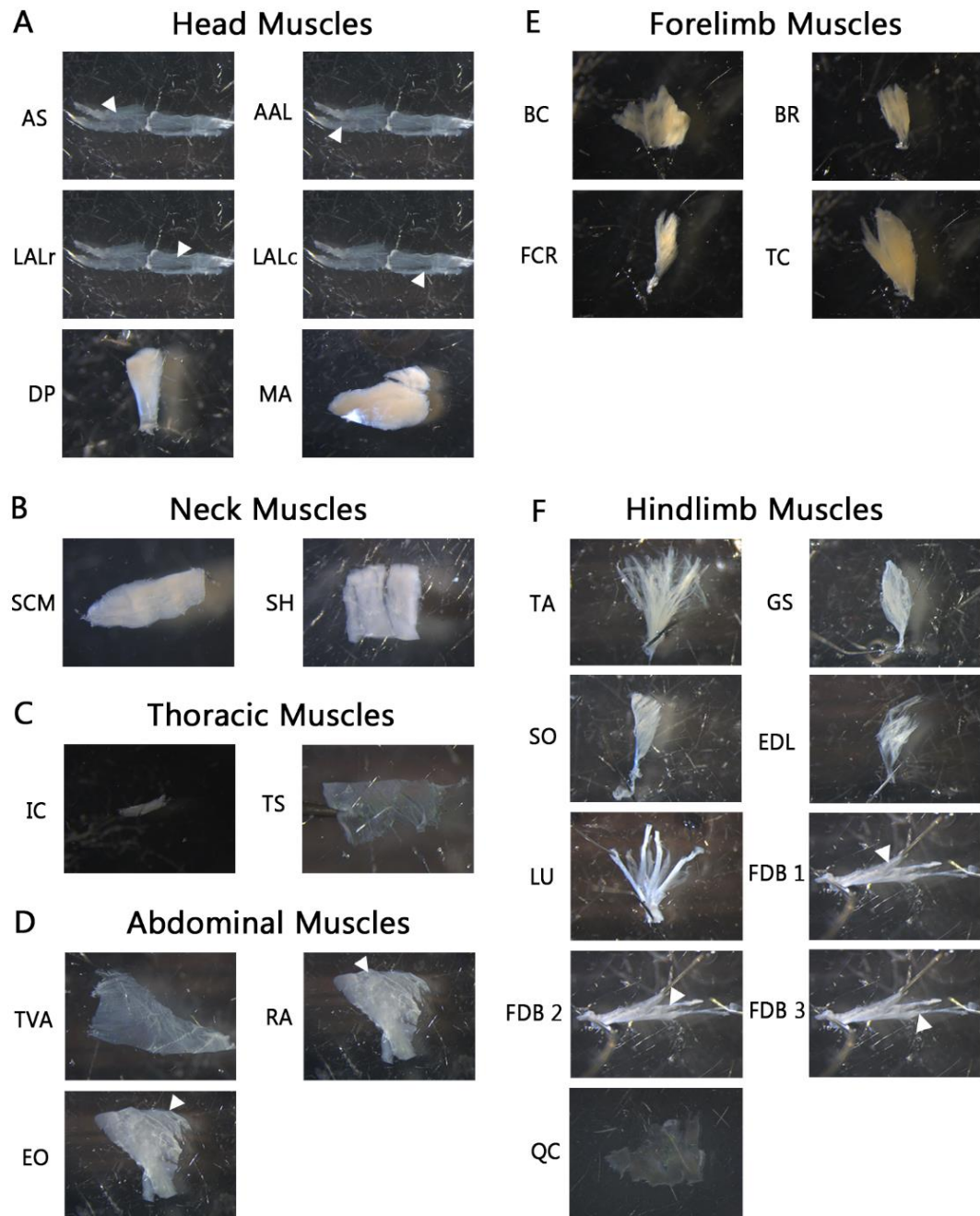


**Figure 2.2: Dissected muscles from different body regions in the *Smn*<sup>2B/-</sup> mouse model.** Representative images of microdissected muscles from different body regions. **(A)** Cranial muscles auricularis superior (AS), adductor auris longus (AAL), levator auris longus rostral band (LALr), levator auris longus caudal band (LALc), digastric posterior (DP) and masseter (MA), **(B)** neck muscles sternocleidomastoid (SCM) and sternohyoid (SH), **(C)** thoracic muscles intercostal muscle (IC) and triangularis sterni (TS), **(D)** abdominal muscles transversus abdominis (TVA), rectus abdominis (RA) and external oblique (EO), **(E)** forelimb muscles biceps (BC), brachialis (BR), flexor carpi radialis (FCR) and triceps (TC), and **(F)** hindlimb muscles tibialis anterior (TA), gastrocnemius (GS), soleus (SO), extensor digitorum longus (EDL), lumbricals (LU), the three heads of flexor digitorum brevis (FDB 1, FDB 2, FDB 3) and quadriceps (QC).

### 2.5.2. NMJ occupancy quantification

NMJ occupancy was quantified manually using a Leica-inverted fluorescent microscope at 40x magnification using filters that allow separate and simultaneous visualisation of green (NF, SV2) and red channels (BTX). All quantifications were undertaken blinded to the genotype of the muscle. All endplates in a given field of view were classified and a minimum of 100 endplates in three fields of view per muscle were quantified. The fields of view were chosen randomly across the muscle using the BTX staining. This ensured unbiased selection of the areas for quantification and a fair representation of endplate occupancy across the muscle. The analysed endplates were classified as vacant, partially innervated and fully occupied, depending on how much of the pre-synaptic terminal covers AChR clusters of motor endplates. Endplates classified as fully occupied, showed the pre-synaptic terminal covering the motor endplate completely. Partially innervated endplates were showing signs of synaptic withdrawal and were only covered 80% or less by the pre-synaptic terminal, while vacant endplates had no remaining pre-synaptic innervation (Figure 2.3A) The number of fully occupied, partially innervated and vacant endplates have been expressed as percentages of the total number of quantified endplates for each muscle. The methods presented here are standard protocols for NMJ quantification (Comley et al., 2016; Courtney et al., 2019; Kline et al., 2017; Mole et al., 2020; Murray et al., 2015, 2013, 2011, 2008; Villalón et al., 2019).

All collected data was assembled in Microsoft Excel and exported to GraphPad Prism 9 for statistical analysis. The data was analysed by two-way ANOVA and use of Sidak correction. Data is presented as mean values  $\pm$  the standard error of the mean (SEM). Data was considered statistically significant when  $p \leq 0.05$ .

Representative example images are projections of z-stacks obtained by a Leica SP8 confocal microscope at 63x magnification with use of the LAS X imaging software (Leica). Z-stack projections were converted to images using ImageJ (<https://imagej.nih.gov/ij/download.html>) and were adjusted for image brightness and contrast using Adobe Photoshop 2020 software. For some

figures, representative images were taken on a Leica DM8 fluorescent microscope at 40x magnification. Quantification of NMJ occupancy in specific sets of muscles was undertaken and provided by Estefania Grados Porro (TVA muscles of IP-administered PACAP or saline treatment; Chapter 4)

### **2.5.3. Level of pre-synaptic swelling quantification**

The level of pre-synaptic swelling of NMJs was quantified manually using a Leica-inverted fluorescent microscope at 40x magnification using filters that allow separate and simultaneous visualisation of green (NF, SV2) and red channels (BTX). As detailed in 2.5.2., all quantifications were undertaken blinded to the genotype of the muscle. Three fields of view were chosen at random and a minimum of 100 endplates per muscle were quantified. The level of pre-synaptic swelling in each NMJ was classified as one of four stages: none, mild, moderate, or severe (Figure 2.3B). Pre-synaptic swelling was classified as none, if there was no evidence of pre-synaptic or axonal swelling. Swelling was classified as mild with some evidence of pre-synaptic or axonal swelling, that does not obscure the endplate, while swelling was graded moderate with evidence of clear axonal swelling and significant pre-synaptic swelling that was beginning to obscure the endplate. Pre-synaptic swelling was classified as severe, if there was significant axonal swelling along the axon present and severe pre-synaptic swelling, which obscured the endplate.

All collected data was assembled in Microsoft Excel and exported to GraphPad Prism 9 for statistical analysis. The data was analysed by two-way ANOVA with Tukey post-hoc test analysis of significance. Data is presented as mean values  $\pm$  SEM. Data was considered statistically significant when  $p \leq 0.05$ .

Representative example images were taken on a Leica DM8 fluorescent microscope at 40x magnification. Quantification of level of pre-synaptic swelling at NMJs was undertaken and provided by lab member Estefania Grados Porro (TVA muscles of IP-administered PACAP or saline treatment; Chapter 4).

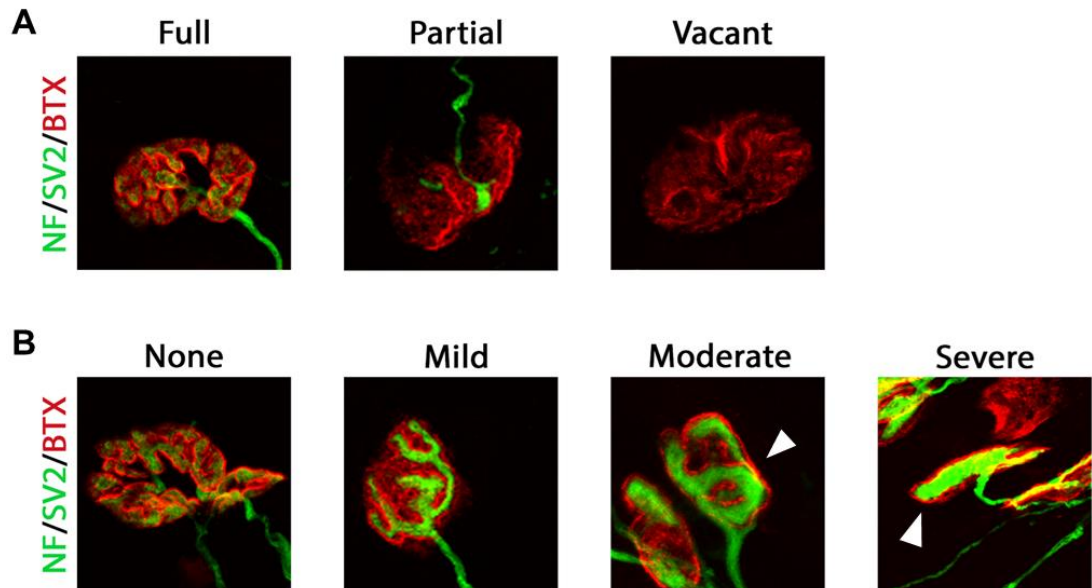


**Figure 2.3: Features of neuromuscular junction pathology for quantification purposes.** **(A)** NMJs labelled with antibodies against neurofilament (NF, green), synaptic vesicle protein 2 (SV2, green) and  $\alpha$ -bungarotoxin (BTX, red). Micrographs show examples of fully occupied (full), partially occupied (partial) and vacant endplates. See section 2.5.2. for full description of criteria used for quantification. **(B)** NMJs labelled with antibodies against neurofilament (NF, green), synaptic vesicle protein 2 (SV2, green) and  $\alpha$ -bungarotoxin (BTX, red). Micrographs show examples of none, mild, moderate, and severe pre-synaptic swelling. Note that white arrowheads point out the NMJ displaying the respective category. See section 2.5.3. for full description of criteria used for quantification.

#### 2.5.4. Immunohistochemistry for spinal cord sections

Spinal cords were immediately dissected from recently sacrificed mice. To dissect the spinal cords, a syringe with needle tip was filled with 1xPBS and inserted into the vertebral canal from the lumbar end. PBS was ejected in the vertebral canal to detach the spinal cord and remove them from the canal into a petri dish. For experiments in Chapter 3, whole spinal cords were prepared for immunohistochemistry. Spinal cords were fixed overnight in 4% paraformaldehyde (PFA, Electron Microscopy Science) in 1xPBS before washing in 1xPBS. They were stored at 4°C in 1xPBS.

To prepare the spinal cords for sectioning and subsequent motor neuron labelling, they were immersed in 30% Sucrose in 1xPBS for 24 hours on a rocking platform at 4°C prior to embedding. Spinal cords were then embedded in 50% optimal cutting temperature compound (OCT)/15% Sucrose in 1xPBS

by snap freezing them in an embedding mould on dry ice. They were stored at -80°C until ready for sectioning. For motor neuron cell body labelling with choline acetyltransferase (ChAT), spinal cord segments T5, T11 and L5 were cryo-sectioned. Each spinal cord segment was transverse sectioned at 10µM thickness. Optimal sectioning conditions were present at temperatures of -20°C for the object head and -22°C for the chamber. Every tenth section was collected onto superfrost plus slides (Thermo Fisher Scientific) and dried for 15 minutes at room temperature. Slides were stored at -20°C in preparation for subsequent labelling with antibodies against ChAT and Nissl.

Staining of slides was performed in the Sequenza Immunostaining Center Slide Rack (Fisher Scientific). All reagents for immunohistochemistry labelling were applied at a volume of 300µL. For ChAT and Nissl labelling, sections were washed in 1xPBS and mounted onto the Sequenza slide rack. Sections were permeabilised in 0.3% Triton X-100 in 1xPBS for 30 minutes and blocked in 0.3% Triton X-100/1% BSA in 1xPBS blocking solution for 60 minutes. Slides were washed twice for 5 minutes in 0.2% Triton X-100 in 1xPBS. Sections were then incubated for 72 hours in ChAT (goat anti-choline acetyltransferase; Merck Millipore; 1:100) primary antibody in 0.3% Triton X-100/1% BSA in 1xPBS at 4 °C. Slides were washed twice in 0.2% Triton X-100 in 1xPBS for 5 minutes. They were incubated in secondary antibody (AlexaFluor 555 Donkey anti-Goat; Life Technologies; 1:250) in 0.3% Triton X-100/1% BSA in 1xPBS for 2 hours at room temperature in the dark. Sections were counterstained for 10 minutes with 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI; Life Technologies; 1:1000) in 0.2% Triton X-100 in 1xPBS and then stained with NeuroTrace® (Nissl 500/525 [green]; Life Technologies; 1:100) for 30 minutes. Sections were washed in 0.2% Triton X-100 in 1xPBS twice for 5 minutes and subsequently mounted in Mowiol®. Slides were stored at 4°C in the dark.

#### **2.5.5. Motor neuron cell body counts in spinal cord**

For work relating to the characterisation of a pattern of selective vulnerability in the *Smn*<sup>2B/-</sup> mouse model of SMA, the total number of motor neuron cell bodies was quantified in different spinal cord segments. Spinal cords were sectioned transversely, and one slide containing every 10<sup>th</sup> section of the

desired spinal cord segment, was stained with ChAT and Nissl. The total number of motor neuron cell bodies within the ventral horn of the spinal cord was quantified by fluorescent microscopy at 20x magnification using a Leica-inverted fluorescent microscope with filters that allow separate and simultaneous visualisation of green (Nissl) and red (ChAT) channels. All quantifications were undertaken blinded to the genotype of the sections. Motor neuron cell bodies were only counted if they could be clearly distinguished from other neuronal material by their large size, irregular shape, and double staining with Nissl and ChAT. They were only counted if the above criteria were met and if they had a visible nucleus and nucleoli. The number of ChAT-positive motor neurons was quantified bilaterally in every tenth section.

Values of motor neuron numbers that were missing due to sectioning artifacts were statistically imputed using the *mice: Multivariate Imputation by Chained Equations in R* (Buuren and Groothuis-Oudshoorn, 2011) package in R studio. The values were assumed as missing at random (MAR) and were imputed using a predictive mean matching (PMM) imputation model. For each missing data point, five different values were predicted. The average from these five predicted values was used as the final imputed value for the following statistical analysis of the total number of motor neuron cell bodies in the ventral horn in each spinal cord segment. Approximately, 12% of all values were imputed.

All collected and imputed data was assembled in Microsoft Excel and exported to GraphPad Prism 9 for statistical analysis. The data was analysed by unpaired Student's t test. Data is presented as mean values  $\pm$  SEM. Data was considered statistically significant when  $p \leq 0.05$ . Representative images were taken on a Leica DM8 fluorescent microscope at 20x magnification.

#### **2.5.6. Extraction of NMJ occupancy data from the literature**

Data on NMJ occupancy in a range of muscles was obtained from previously published studies in four mouse models of SMA. Studies detailing NMJ occupancy quantifications in *Smn*<sup>-/-</sup>; *SMN2* (Murray et al., 2010a, 2008; Thomson et al., 2012), Taiwanese (Buettner et al., 2021; Lin et al., 2016), *SMNΔ7* (Buettner et al., 2021; Comley et al., 2016; Ling et al., 2012; Murray

et al., 2008) and *Smn*<sup>2B/-</sup> (Murray et al., 2015) mouse models were utilised. Graphs showing the percentage of fully occupied endplates were exported from the respective studies into Adobe Photoshop (version 21.2.0). A grid overlay was aligned to each graph's axes to allow exact measurements of the mean percentage of fully occupied motor endplates in each muscle of interest.

Following the acquisition of the occupancy data, results were pooled by mouse model and muscles were allocated to seven classification categories according to their percentage of fully occupied endplates at the end-stage of the disease. Each classification category was assigned a different colour (ranging from green, representing the muscles with minimal synaptic loss, to magenta representing the muscles with large amounts of synaptic loss) for representation in figures. The first six classification categories each include the muscles with percentages of fully occupied endplates within a 10% range, forming categories from 100 to 41% fully occupied endplates. The last category includes all muscles that have 40% or less fully occupied endplates at disease end-stage. All graphs were created using GraphPad Prism8.

#### **2.5.7. Fluorescent intensity in PACAP treated brain and liver**

*Smn*<sup>2B/-</sup> animals were treated with 182.49mg/kg of FITC-conjugated PACAP27 or 251.8mg/kg of FITC-conjugated PACAP38 via IP injection daily from P5 to end of life. To detect any amelioration in PACAP levels after the treatment, the fluorescent intensity of PACAP levels in the liver and brain was measured. Livers and brains were immediately dissected from recently sacrificed mice. Brains were separated along the midline, and one half of the brain, along with the liver samples, was fixed overnight in 4% PFA. Samples were stored in 1xPBS at 4°C in preparation for fluorescent intensity measurements.

Brain and liver samples were equilibrated in 30% Sucrose in 1xPBS overnight, before embedding in 50% OCT/15% Sucrose in 1xPBS by snap-freezing them on an isopentane and dry ice mixture. The isopentane/dry ice mixture was used to accelerate the freezing process in comparison to only using dry ice, therefore minimising freezing artifacts in the tissues. Tissues were cryo-sectioned at 12µm thickness and collected onto superfrost plus slides. Slides

were dried for 15 minutes at room temperature and stored at -20°C. Slides were washed with 1xPBS, stained with DAPI (1:1000) in 1xPBS for 5 minutes and again washed with 1xPBS. Slides were mounted using Mowiol®.

Images were taken using an inverted fluorescent Leica DM8 microscope at 40x magnification, imaging the green and blue channels. One image was acquired on four sections per animal for each tissue and each genotype. To quantify the amount of PACAP present in livers and brains of treated *Smn*<sup>2B/-</sup> and control animals, the fluorescent intensity of the FITC-conjugated PACAP27 and PACAP38 was quantified using ImageJ. The average fluorescent background intensity of images was measured in the green channel only. The measured intensity values were normalised by dividing each value from PACAP treated animals by the values of their respective saline treated control group. All quantifications were undertaken blinded to the genotype of the sections.

Collected data was assembled in Microsoft Excel and exported to GraphPad Prism 9 for statistical analysis. For each genotype a n number of n=3 for liver samples and a n=3 for brain samples has been analysed. The data was analysed by Kruskal-Wallis multiple comparisons test with Dunn's correction. Data is presented as mean values  $\pm$  SEM. Data was considered statistically significant when  $p \leq 0.05$ . Sectioning, staining, imaging and quantification of liver and brain samples was undertaken and provided by MSc student Estefania Grados Porro.

#### **2.5.8. AAV9-PACAP intensity quantification in spinal cords**

*Smn*<sup>2B/-</sup> animals and their littermate controls were treated with a low dose  $3.33 \times 10^{10}$  VG/mL AAV9-PACAP-GFP or saline at P1 or with a high dose of  $8.07 \times 10^{12}$  VG/mL AAV9-PACAP-GFP or saline at P2 or P3 via a single ICV injection. To detect any amelioration in PACAP levels after the treatment, the fluorescent intensity of PACAP levels in the spinal cord was measured in PACAP treated and saline treated *Smn*<sup>2B/-</sup> mice. Spinal cords were immediately dissected from recently sacrificed mice. All tissues were fixed

overnight in 4% PFA. Samples were stored in 1xPBS at 4°C in preparation for PACAP intensity measurements.

Spinal cords were immersed in 30% Sucrose in 1xPBS for 24 hours on a rocking platform at 4°C prior to embedding. Spinal cords were then embedded in 50% optimal cutting temperature compound (OCT)/15% Sucrose in 1xPBS by snap freezing them in an embedding mould on dry ice. They were stored at -80°C until ready for sectioning. For PACAP labelling of MN cell bodies, the mid-thoracic spinal cord region was cryo-sectioned. Spinal cords were transverse sectioned at 10µM thickness. Optimal sectioning conditions were present at temperatures of -20°C for the object head and -22°C for the chamber temperature. Sections were collected onto superfrost plus slides and dried for 15 minutes at room temperature. Slides were stored at -20°C in preparation for subsequent labelling with antibodies against PACAP and Nissl.

All reagents for immunohistochemistry labelling were applied at a volume of 300µL. Slides were washed in 1xPBS and mounted into the Sequenza Immunostaining Center Slide Rack (Fisher Scientific) slide rack. Sections were permeabilised in 0.3% Triton X-100 in 1xPBS for 30 min and blocked in 0.3% Triton X-100/1% BSA in 1xPBS blocking solution for 60 min. Sections were then incubated for 24 hours in PACAP27/38 (Pituitary adenylate cyclase-activating peptide 27/38; Bioss USA; 1:250) primary antibody in 0.3% Triton X-100/1% BSA in 1xPBS at 4 °C. Slides were washed twice in 0.2% Triton X-100 in 1xPBS for 5 minutes. They were incubated in secondary antibody (AlexaFluor 555 Goat anti-Rabbit; Life Technologies; 1:250) in 0.3% Triton X-100/1% BSA in 1xPBS for 2 hours at room temperature in the dark. Sections were counterstained for 10 minutes with DAPI (Life Technologies; 1:1000) in 0.2% Triton X-100 in 1xPBS and then stained with NeuroTrace® (Nissl 500/525 [green]; Life Technologies; 1:100) for 30 minutes. Sections were washed in 0.2% Triton X-100 in 1xPBS twice for 5 minutes and subsequently mounted in Mowiol®. Slides were stored at 4°C in the dark.

Images were taken using an inverted fluorescent Leica DM8 microscope, imaging the green, red and blue channels. Representative images of each

ventral horn of the spinal cord were acquired for all samples and genotypes (9 images per sample for low dose PACAP treated animals, and 3 images per sample for high dose PACAP treated animals). To quantify the amount of PACAP present in thoracic spinal cords of PACAP and saline treated *Smn*<sup>2B/-</sup> and control animals, the mean grey value of the motor neurons present in the ventral horn was quantified using ImageJ. The mean grey value of 10 motor neurons in each image was measured in the red channel only (labelled against PACAP27/38 antibody). The measured intensity values were normalised by dividing each value from PACAP treated animals by the values of their respective saline treated control group.

Collected data was assembled in Microsoft Excel and exported to GraphPad Prism 9 for statistical analysis. For low dose PACAP and saline treatment, an n-number of n=1 has been analysed for each genotype. For high dose PACAP samples, a n number of n=3 for saline treated and PACAP treated controls, a n=1 for saline treated *Smn*<sup>2B/-</sup> and a n=4 for PACAP treated *Smn*<sup>2B/-</sup> samples has been analysed. The data was analysed by Kruskal-Wallis multiple comparisons test with Dunn's correction. Data is presented as mean values  $\pm$  SEM. Data was considered statistically significant when  $p \leq 0.05$ . Sectioning, staining, imaging, and quantification of spinal cord samples was undertaken and provided by MSc student Christy Ip.

## **2.6. Library preparation for single cell LCM-sequencing**

### **2.6.1. Tissue collection and dissection of brainstems**

Brainstems were immediately dissected from recently sacrificed SMN $\Delta$ 7 mice and their control littermates. In the SMN $\Delta$ 7 mouse model, both heterozygous animals (*Smn*<sup>+/-</sup>; *SMN2*<sup>2copy</sup>; *SMN $\Delta$ 7*<sup>2copy</sup>; aka 'Het') and homozygous animals (*Smn*<sup>+/+</sup>; *SMN2*<sup>2copy</sup>; *SMN $\Delta$ 7*<sup>2copy</sup>; aka WT), were previously shown to not show a SMA phenotype and can therefore be considered as control littermates (Monani et al., 2000). However, for these experiments only Het animals were used as littermate control to keep the genetic variation within the control group as small as possible. Tissues from SMN $\Delta$ 7 and control animals were collected at three time points: P2 as pre-symptomatic, P5 as early symptomatic and P9 as late symptomatic. Brains were removed by opening up the skull and

detaching the brains from the base of the skull by severing the olfactory nerves. Brainstems were microdissected by detachment from the surrounding brain regions and were cleaned by removing any leftover tissue from the cerebellum and the cervical spinal cord.

### **2.6.2. Embedding and sectioning of brainstems**

After dissection, the brainstems were immediately embedded with their ventral side facing downwards in OCT and snap frozen on dry ice to avoid RNA degradation. They were stored at -80°C until ready for sectioning. Tissue blocks were kept in -22°C cold cryostat chamber for 30 minutes prior to sectioning to adjust to cutting temperature. Slides and slide boxes for storage of sectioned slides were also kept in cryostat chamber for 30 minutes prior to sectioning to pre-cool and avoid freezing artefacts while sectioning. Brainstems were cryo-sectioned at 10µm thickness. Optimal sectioning conditions were present at temperatures of -20°C for the object head and -22°C for the chamber. Every section of the brainstems was collected onto 1.0 polyethylene naphthalate (PEN) membrane slides (Carl Zeiss), fitting four sections per slide. Readily processed slides with collected sections were kept in the cryostat chamber at -22°C until sectioning was completed. Slide box with sections was transported on dry ice, and slides were stored at -80°C in preparation for subsequent HistoGene labelling and LCM.

### **2.6.3. HistoGene staining**

In preparation for laser capture microdissection (LCM) of brainstems as described in Chapter 5, slides were labelled with HistoGene. Only one slide at a time was stained following a previously published method (Nichterwitz et al., 2018). All equipment and working spaces were cleaned with 70% ethanol and RNase Zap™ wipes (Invitrogen) prior to the staining process and slide was only handled using forceps. One 50mL falcon tube each was prepared with Ultrapure water (Invitrogen) only, 75%, 95% and 99.5% ethanol in Ultrapure water.

Slide was transported in a slide box on dry ice from the -80°C freezer to the staining set up. Slide was removed from the slide box and placed flat, with the

PEN membrane and sections facing upwards, on the staining set up. Slide was defrosted for 30 seconds and then placed in the tube with 75% ethanol for 30 seconds to fix the tissue sections. The slide was washed in Ultrapure water for 30 seconds and the remaining water was removed by carefully tipping the bottom of the slide against the lint free tissues. Slide was then placed flat on the staining set up and 200µL HistoGene (Arcturus) staining solution was pipetted onto the sections, incubating it for 60 seconds. The remaining stain was carefully removed, and the slide was washed once again in Ultrapure water for 30 seconds. The slide was dehydrated in a series of alcohols, being placed 30 seconds each in 75%, 95% and 99.5% ethanol in Ultrapure water. The readily stained slide was then stored in a clean and RNase free slide box and used for subsequent LCM for a maximum of 3 hours. All steps of the protocol were performed while maintaining an RNase free environment.

#### **2.6.4. Single cell motor neuron collection via LCM**

Single motor neurons from the CN VII from SMN $\Delta$ 7 and control animals were collected by laser capture microdissection using a Leica LMD6000 system. 30 minutes prior to the start of the motor neuron collection, the LCM system (microscope, laser, computer, and LMD software) were started and the ventilation in the LCM chamber was turned on to rid the chamber of any dust or previous tissue remnants. Protective clothing consisting of lab coat, hairnet, shoe covers, and gloves was worn at all times while operating the LCM system to minimise the transmission of other RNA to the samples and the LCM system. All equipment and working spaces were cleaned with 70% ethanol and RNase Zap<sup>TM</sup> wipes prior to utilising the LCM system.

Slide was taken out of the slide box and carefully placed face down into the slide holder for the motorised stage. Static was removed from the slide holder with the inserted slide using a Static Eliminator (Plas-Labs; 800-AS/SPI, "Counter SPI", Work Station Ionizer) for 10 minutes. Four RNase/DNase-free 0.2mL PCR tubes (Axygen) at a time were placed into the cap holders of the collector device from the LCM system. 3µL of modified lysis buffer (Table 2.4; lysis buffer with half the amount of 10µM Smarter\_oligo-dTVN [Integrated DNA technologies] and without ERCC\_Spike-Ins, 4<sup>e-5</sup> dilution [Fisher Scientific];

adapted from (Schweingruber et al., 2023)) were pipetted into each PCR tube cap. The slide holder and collector device were inserted into the LCM system and the Leica LMD programme was used to generate an overview of the slide at 2.5x magnification for easier navigation. Subsequently, a more detailed 10x magnification scan was produced of each section that was used for collection of single motor neurons cell bodies.

To start the LCM of single motor neuron cell bodies, the facial nucleus (CN VII) was identified on a section of interest. On 40x magnification, motor neurons of interest were microdissected using the LMD software. A cutting outline was drawn closely around the outline of the motor neuron soma of interest to avoid contamination with other cell types from the surrounding tissue. A single motor neuron was cut out of the section with the laser and captured in the lysis buffer filled cap of a 0.2mL PCR tube in the collector device (Figure 2.4). After one cell each was captured in all four PCR tube caps in the collector device, the tubes were unloaded from the device, closed and immediately snap-frozen on dry ice. The remaining motor neurons for each sample, genotype and time point were captured following the same steps. For each genotype and time point, a number of  $n=3$  animals, was subject to capturing 44-60 single motor neurons per animal (Table 2.5). Each slide was used for a maximum duration of 3 hours for LCM capturing post HistoGene staining. All steps of the protocol were performed while maintaining an RNase free environment. Samples were stored at  $-80^{\circ}\text{C}$  until further processing.

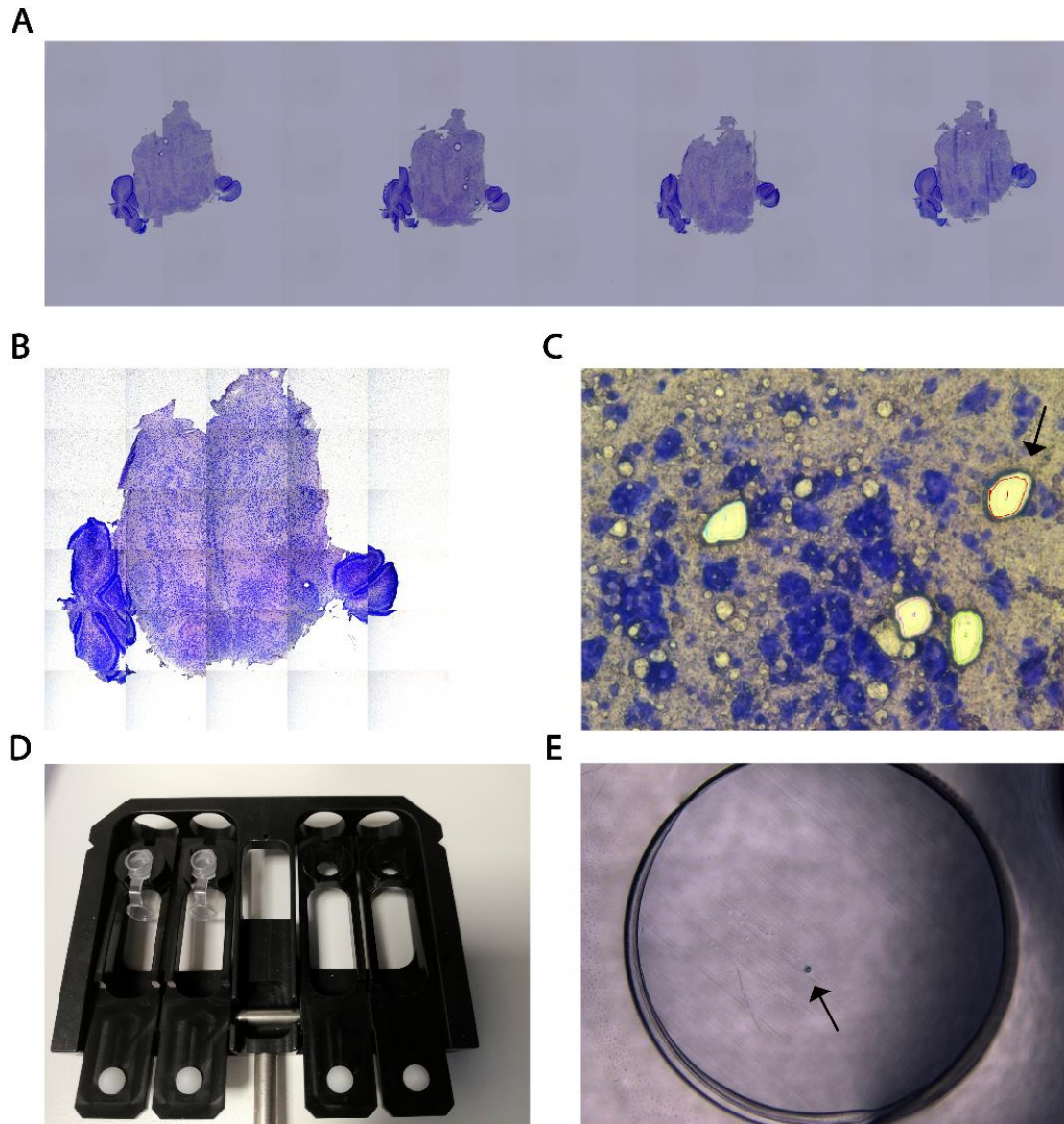


**Figure 2.4: Motor neuron collection with laser capture microdissection.** Representative pictures of **(A)** the overview scan of brainstem sections on a 1.0 PEN membrane slide taken at 2.5x magnification, **(B)** a brainstem section scan closeup taken at 10x magnification, **(C)** a microdissected single motor neuron, cut out with the laser using Leica's LMD software at 40x magnification, **(D)** the collector device, showing how the lids of 0.2mL PCR tubes are secured for collection of single motor neurons, and **(E)** a single collected motor neuron in the lid of a 0.2mL PCR tube at 5x magnification.

**Table 2.4: Modified lysis buffer reagents for scLCM-seq**

| Reagent                      | 1x (μL) | 110x (μL) |
|------------------------------|---------|-----------|
| 0.2% Triton X-100 + 0.5% RRI | 2.02    | 222.2     |
| 10μM Smarter_oligo-dTVN      | 0.25    | 27.5      |
| 25 mM dNTP                   | 0.4     | 44.0      |
| 100 mM DTT                   | 0.03    | 3.3       |
| Total                        | 2.7μL   | 297μL     |

**Table 2.5: Summary of LCM collected tissues**

| ID | Age | Genotype | Number of LCM Samples | Number of Libraries |
|----|-----|----------|-----------------------|---------------------|
| A  | P5  | Control  | 36                    | 0                   |
| B  | P5  | Control  | 44                    | 44                  |
| C  | P5  | SMNΔ7    | 44                    | 44                  |
| D  | P5  | SMNΔ7    | 60                    | 60                  |
| E  | P5  | SMNΔ7    | 52                    | 52                  |
| F  | P5  | Control  | 60                    | 60                  |
| G  | P5  | Control  | 60                    | 60                  |
| H  | P9  | Control  | 65                    | 65                  |
| I  | P9  | Control  | 64                    | 64                  |
| J  | P9  | Control  | 58                    | 58                  |
| K  | P9  | SMNΔ7    | 60                    | 60                  |
| L  | P9  | SMNΔ7    | 64                    | 64                  |
| M  | P9  | SMNΔ7    | 60                    | 60                  |
| N  | P2  | Control  | 60                    | 60                  |
| O  | P2  | Control  | 60                    | 60                  |
| P  | P2  | Control  | 60                    | 60                  |
| Q  | P2  | SMNΔ7    | 60                    | 60                  |
| R  | P2  | SMNΔ7    | 60                    | 60                  |
| S  | P2  | SMNΔ7    | 60                    | 56                  |

### **2.6.5. cDNA library preparation**

To produce cDNA libraries from the LCM collected single motor neurons, first and second strand synthesis, followed by cDNA library purification needed to be performed. All cDNA libraries were prepared as described in (Schweingruber et al., 2023). Libraries were prepared by processing 96 samples at a time, making sure to include some samples from across genotypes and all three time points in each processed batch. This was done to minimise preparation bias and artifacts in RNA-seq analysis when processing samples from different origin. All libraries were prepared in a fume hood and all equipment and working spaces were cleaned with 70% ethanol and RNase Zap<sup>™</sup> wipes prior to use. The first strand synthesis reaction mix was prepared on ice immediately before use (Table 2.6).

Lysed single motor neuron samples were transported on dry ice and thawed on a cold metal stand to minimise RNA degradation. Cells were collected at the bottom of the tube by centrifuging for 15 seconds at <2000g. To denature the samples for the reverse transcription process, they were placed in a thermocycler at 72°C for 3 minutes. Samples were then cooled on ice for 2 minutes, followed by brief centrifuging to collect the samples. 2.76µL of first strand synthesis reaction mix were pipetted into each tube with single motor neuron lysate using a multichannel pipette. Reaction reagents were combined by centrifuging for 15 seconds at <2000g. Samples were placed in the thermocycler and a programme (Table 2.7) was run to achieve the reverse transcription with template switching of the samples.

**Table 2.6: First strand synthesis reaction mix**

| Reagent                | 1x (μL) | 110x (μL) |
|------------------------|---------|-----------|
| 5x SSII buffer         | 1.0     | 110.0     |
| 5 M Betaine            | 1.0     | 110.0     |
| 100 mM DTT             | 0.25    | 27.5      |
| 1 M MgCl <sub>2</sub>  | 0.035   | 3.9       |
| 200 units/μL SSII RT   | 0.25    | 27.5      |
| 40 units/μL RRI        | 0.125   | 13.8      |
| 100 μM Smarter_TSO-LNA | 0.1     | 11.0      |
| Total                  | 2.76μL  | 303.6μL   |

**Table 2.7: Reverse transcription with template switching temperature cycle**

| Step Number                 | Action                        | Temperature (°C) | Time          |
|-----------------------------|-------------------------------|------------------|---------------|
| 1.                          | Warm up                       | 42°C             | Infinite hold |
| 2.                          | Initial reverse transcription | 42°C             | 90 minutes    |
| 3.                          | RNA unfolding                 | 50°C             | 2 minutes     |
| 4.                          | Template switch               | 42°C             | 2 minutes     |
| <b>Repeat steps 3-4 10x</b> |                               |                  |               |
| 5.                          | Inactivation                  | 70°C             | 15 minutes    |
| 6.                          | Final hold                    | 4°C              | Infinite hold |

The second strand synthesis reaction mix was prepared on ice immediately before use (Table 2.8). The first strand synthesis reaction samples were removed from the thermocycler and centrifuged for 15 seconds at <2000g. Samples were placed in an ice old metal stand and 7.5μL of the second strand synthesis reaction mix was pipetted into each sample tube using a multichannel pipette. Reagents were combined with samples by brief centrifuging. Samples were placed into the thermocycler and a second strand synthesis and amplification programme (Table 2.9) was run.

**Table 2.8: Second strand synthesis reaction mix**

| Reagent             | 1x (μL) | 110x (μL) |
|---------------------|---------|-----------|
| Ultrapure water     | 1.15    | 126.5     |
| 2 μM SMARTer_ISPCR  | 0.1     | 11.00     |
| 2x KAPA HiFi HS mix | 6.25    | 825.0     |
| Total               | 7.5μL   | 687.5μL   |

**Table 2.9: Second strand synthesis and amplification temperature cycle**

| Step Number                 | Action               | Temperature (°C) | Time          |
|-----------------------------|----------------------|------------------|---------------|
| 1.                          | Warm up              | 98°C             | Infinite hold |
| 2.                          | Initial denaturation | 98°C             | 3 minutes     |
| 3.                          | Denaturation         | 98°C             | 20 seconds    |
| 4.                          | Annealing            | 67°C             | 15 seconds    |
| 5.                          | Elongation           | 72°C             | 6 minutes     |
| <b>Repeat steps 3-5 24x</b> |                      |                  |               |
| 6.                          | Final elongation     | 72°C             | 5 minutes     |
| 7.                          | Final hold           | 12°C             | Infinite hold |

After completion of the first and second strand synthesis, the cDNA libraries need to be purified using magnetic SPRI beads in preparation for quality control of the samples. The SPRI beads (19.5% PEG-8 k) were equilibrated to room temperature for 20 minutes prior to purification. Once equilibrated, 12.5μL beads per well were pipetted into a 96-well deep well plate (Axygen; 96-well clear V-bottom) using a multichannel pipette. Samples were removed from the thermocycler and placed onto an ice-cold metal stand. Samples were centrifuged for 15 seconds at <2000g to collect at the bottom of the tubes. Samples were removed from the original collection tubes using a multichannel pipette and transferred column by column into the deep well plate with the SPRI beads. Samples were mixed with the beads by pipetting up and down 10x. The plate was covered, and the samples were incubated for 8 minutes at room temperature to allow the magnetic beads to bind to the samples. The covered plate was then placed onto a magnetic stand for 5 minutes to collect

the beads in a pallet to the sides of the wells. The supernatant was discarded without disturbing the beads and the beads palette was carefully washed with 100µL of 80% ethanol, before discarding the ethanol. The beads were once again collected on the magnetic stand and were dried uncovered for 10 minutes. 13µL elution solution (1% 1M Tris-HCl pH 8.0 [Fisher Scientific] in Ultrapure water) were pipetted into each well using a multichannel pipette before removing the 96-well plate from the magnetic stand. Beads were resuspended in the elution solution and were incubated covered at room temperature for 5 minutes to release the samples from the beads. After incubation, the plate was once again placed on the magnetic stand to collect the beads for at least 2 minutes. Using a multichannel pipette, 12µL of the eluate from each sample were transferred to a 96-well storage plate. The final purified single cell cDNA libraries were stored at -20°C until cDNA quantification and quality control of the samples was performed.

#### **2.6.6. cDNA quantification, quality control and sample dilutions**

To determine the quality and the concentrations of the single cell cDNA libraries, two methods were utilised on the prepared libraries. To quantify the cDNA amount in each single cell library, the cDNA concentration of the individual samples was measured with a Qubit assay system based on a fluorescent dsDNA intercalating dye (Qubit dsDNA High-Sensitivity assay systems; Fisher Scientific). Following the protocol from Schweingruber et al., 2023, 49µL of Qubit 1x dsDNA High-Sensitivity assay solution were pipetted in each sample well of a black 96-well assay plate (Fisher Scientific). 1µL of each single cell library was transferred into the assay solution of the assay plate. For the assay calibration, wells with two different standards were prepared using four replicates. 47.5µL of Qubit 1x dsDNA High-Sensitivity assay solution were pipetted in each standard well of the black assay plate. 2.5µL each of standard 1 (0ng/µL dsDNA, background) and standard 2 (10ng/µL dsDNA, upper boundary) were transferred into the assay solution. Following preparation of the samples and standard on the 96-well assay plate, the fluorescent signal from the dsDNA assay system was recorded on a microplate spectrofluorometer (SpectraMax, Biotec). The spectrofluorometer was set to excitation at 485nm and emission at 530nm. Fluorescent read

values were recorded using the Soft Max Pro software (Molecular Devices) and were exported and assembled in Microsoft excel. The expected range of cDNA concentrations was 0.5 to 5.0ng/μL cDNA libraries. Concentrations of the single cell libraries were calculated with the dilution factor  $f_{\text{Dilution}} = 50$  and the average values of the standards, by utilising the following equation as outlined in Schweingruber et al., 2023:

$$\text{Conc} = f_{\text{Dilution}} \times 0.5 \times \left( \frac{F_{\text{Sample}} - F_{\text{Standard 1}}}{F_{\text{Standard 2}} - F_{\text{Standard 1}}} \right) \text{ ng}/\mu\text{l}$$

Following the measurement of cDNA concentrations of single cell libraries, each single cell cDNA libraries was diluted with Ultrapure water to a final cDNA concentration of 1ng/μL in preparation for quality control and tagmentation. Quality control of cDNA libraries was performed utilising a Bioanalyzer 2100 (Agilent) for chip-based capillary electrophoresis. 10 samples from each 96-well plate (concentration of 1ng/μL) were pooled together, using 1μL from each sample. This was to assess these pools for the overall cDNA quality in the LCM motor neuron sample set. To assess the molecular size distribution of the single cell cDNA libraries, a High Sensitivity DNA Kit (Agilent) was used on a Bioanalyzer 2100. The instructions of the High Sensitivity DNA Kit were followed to load the DNA chip for the Bioanalyzer. In brief, the gel for the loading chip was prepared by pipetting blue dye into the gel mix from the kit. The gel was mixed well, transferred to a column tube and centrifuged at  $2240 \text{ g} \pm 20\%$  for 15 minutes. To load the DNA chip for chip-based capillary electrophoresis, the chip was inserted into the supplemented metal holder with syringe. 9μL prepared gel were loaded in the marked well on the chip. The gel was distributed on the chip by pressing the plunger of the metal holder and keeping it in place for 60 seconds. Upon release of the plunger, the chip was loaded with 9μL gel in the remaining three marked positions. 1μL ladder was added to the chip and each sample well was loaded with 5μL marker. 1μL of each cDNA library pool was added to a sample well. The DNA chip was vortexed for 1 minute and inserted into the Bioanalyzer 2100 to assess the molecular size distribution of the single cell cDNA libraries. Molecular size

profiles from each library pool were collected using the 2100 Bioanalyzer Expert Software (Agilent) and exported for further evaluation.

#### **2.6.7. Library tagmentation and RNA-sequencing**

After successful cDNA quantification and quality control verification of the cDNA libraries using the Bioanalyzer 2100, the quality of the sequencing data from the generated single cell cDNA libraries was assessed. 24 samples were chosen for low throughput sequencing on an Illumina MiSeq platform at the National Genomics Infrastructure (NGI) facility in Stockholm, Sweden. This platform can generate 1 to 10 million reads per sample and allows for an affordable test run and quality control of the sequencing data.

24 purified and readily prepared single cell cDNA libraries with a wide range of initial cDNA concentrations, from across all three time points, and different genotypes were chosen for tagmentation and MiSeq sequencing. Tagmentation of these samples was performed by Irene Mei, member of the Hedlund laboratory. Nextera XT reagents were used to uniquely barcode and fragment the libraries and tagmentation was performed following section 3.7 from Schweingruber et al., 2023. An enrichment PCR was performed on the diluted single cell libraries (concentration 1ng/μL), barcoding them by unique combinations of Illumina-compatible i5/i7 indices. Following PCR, 1μL of each indexed single cell library was used to perform cDNA quantification with a Qubit assay system as described in section 2.6.6. of this thesis. Subsequently, equal amounts of cDNA from each indexed single cell library were combined to produce a sequencing pool. The sequencing pool was then purified as described in section 2.6.5., but by using 24% SRPI beads (0.8:1.0 beads to DNA ratio) and eluting the sequencing pool in 50μL elution solution. The 24 samples were pooled in one lane of a flow cell. 51-bp single-end sequencing was performed on a MiSeq sequencing platform resulting in a read depth of up to 800.000 reads per sample.

## 2.7. RNA-sequencing analysis

### 2.7.1. Transcriptional analysis of CN VII motor neurons in two mouse models of SMA

Transcriptional analysis was performed on differentially vulnerable motor neurons from the facial nucleus (cranial nucleus VII [CN VII]) in the SMN $\Delta$ 7 and the *Smn*<sup>2B/-</sup> mouse model of SMA. Three previously published RNA-sequencing data sets (Table 2.10) were re-analysed and cross-compared. The first data set comprised of raw data from facial motor neurons for *Smn*<sup>2B/-</sup> mice and their controls which was obtained via a kind agreement from the Kothary laboratory and was previously published in Murray et al., 2015. The raw data from the second data set, was from facial motor neurons for SMN $\Delta$ 7 mice and their controls. It was obtained from the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>; Hedlund laboratory, GEO accession number: GSE115706) and was previously published in Nichterwitz et al., 2020. The third data set comprised of raw data from the thoracic spinal cord (tSC) for *Smn*<sup>2B/-</sup> mice and their controls which was obtained via a kind agreement from the Kothary laboratory and was previously published in Murray et al., 2015.

**Table 2.10: Summary of raw data sets for cross-model RNA-seq of CN VII**

|            | Model                      | Population | Time point | Publication              |
|------------|----------------------------|------------|------------|--------------------------|
| Data set 1 | <i>Smn</i> <sup>2B/-</sup> | CN VII     | P10        | Murray et al., 2015      |
| Data set 2 | SMN $\Delta$ 7             | CN VII     | P2         | Nichterwitz et al., 2020 |
| Data set 3 | <i>Smn</i> <sup>2B/-</sup> | tSC        | P10        | Murray et al., 2015      |

The RNA-seq analysis was started by mapping the raw data reads from all data sets to the 10-mm mouse genome assembly. To achieve a similar sequencing depth and mapping ratios across all samples of all three data sets, the raw fastq data from the *Smn*<sup>2B/-</sup> data sets were down-sampled. Data quality control was performed by generating principal component analysis (PCA) plots and heatmaps. PCA plots considered the complete sets of genes and heatmaps considered DESeq counts for the overall gene set. Differential gene

expression analysis was performed on data sets from both models utilising the DESeq2 (v. 1.30.1) package (Love et al., 2014) in R studio. Only genes that showed a log2 fold change difference of 1.5 between SMA and control animals, and genes with an adjusted p-value of  $p \leq 0.05$  were considered for the transcriptional analysis. Lists of DEGs were produced for the *Smn*<sup>2B/-</sup> and SMNΔ7 data sets. DEGs that were overlapping between different data sets were obtained and discarded. All subsequent analyses were then performed on the remaining, not overlapping, DEGs of the *Smn*<sup>2B/-</sup> CN VII data set only. An over-representation analysis was performed on GO terms associated with the *Smn*<sup>2B/-</sup> DEGs using clusterProfiler (v. 3.18.1). Next, heatmaps of the z scores derived from normalised gene counts were generated, to visualise the expression profile of the GO terms associated genes between samples of both data sets. To retrieve networks of protein-protein interaction, a STRING analysis (<https://string-db.org/>; v. 11.5; Szklarczyk et al., 2017) was performed on all DEGs from the *Smn*<sup>2B/-</sup> samples (433 genes were used as input). Interactions from database and experimental evidence were included in the STRING analysis. A maximum of 5 interactions for each node of the first shell were considered, along with a medium confidence for interaction score up to 0.04. All data was analysed and assembled using R Studio software. All above-described transcriptional analyses were performed in collaboration with Irene Mei from the Hedlund laboratory at Stockholm University (Chapter 4).

### **2.7.2. Transcriptional analysis of wildtype motor neurons in two mouse models of SMA**

Transcriptional analysis was performed on differentially vulnerable motor neurons from control (wildtype; WT) littermates of the SMNΔ7 and the *Smn*<sup>2B/-</sup> mouse model of SMA. Two previously published RNA-sequencing data sets were re-analysed and cross-compared. The first data set contained data from differentially vulnerable motor neurons from *Smn*<sup>2B/-</sup> mice and their controls. It was previously published in Murray et al., 2015 and was obtained via a kind agreement from the Kothary laboratory. The raw data from the second data set, contained data from differentially vulnerable motor neurons from SMNΔ7 mice and their controls. It was obtained from the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>; Hedlund laboratory, GEO accession

number: GSE115706) and was previously published in Nichterwitz et al., 2020. To perform a cross-model transcriptional analysis, differentially vulnerable motor neuron data from wildtype animals was compared at time-points that coincides with symptom onset in both SMA mouse models (P2 in Nichterwitz data set; P10 in Murray data set). Motor neuron populations that are differentially vulnerable in the *Smn*<sup>2B/-</sup> (Murray data set) and the SMN $\Delta$ 7 mouse model (Nichterwitz data set), were compared in neurologically normal mice: resistant facial nucleus (CN VII) motor neurons and vulnerable thoracic spinal cord (tSC) motor neurons from the Murray data set were compared to resistant oculomotor (CN III), trochlear (CN IV) and hypoglossal nuclei (CN XII) motor neurons, and vulnerable spinal cord motor neurons from the Nichterwitz data set.

Dr Sang Seo kindly performed and provided normalisation, quality control, annotation and DESeq2 analysis in R on the raw data of the SMN $\Delta$ 7 data set, while a list of differentially expressed genes from the *Smn*<sup>2B/-</sup> mouse model was provided by our lab. All differentially expressed genes with an adjusted p-value of  $p > 0.05$  were eliminated from both data sets. Microsoft Excel was then used to identify commonly differentially expressed genes between both data sets. These genes showed the same directional changes and were present in both RNA-seq data sets. A gene was considered significantly differentially expressed with a p-value of  $p \leq 0.05$  and a greater than  $\pm 1.5 \log_2$  fold change in expression. Top candidates of interest were genes with the largest  $\log_2$  fold change in a common direction. To reveal the enrichment and functional clustering of genes, all differentially expressed genes present in both screens, independent of their fold change and significance, were submitted to the Database for Annotation, Visualisation and Integrated Discovery (DAVID). The gene lists were submitted to the DAVID 6.8 *Mus musculus* database and analysed using the Functional Annotation Clustering tool in DAVID Bioinformatics Resources (version 6.8; <https://david.ncifcrf.gov/summary.jsp>) to produce a list of functional annotations. Functional clusters from this analysis are ranked by an enrichment score from DAVID. Functional clusters with an enrichment score (ES) of  $> 1.3$  were considered to be statistically significant, as this in DAVID is the equivalent to  $p \leq 0.05$  (Huang et al., 2007).

## Chapter 3

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### Investigating neuromuscular junction pathology and patterns of selective vulnerability in four mouse models of SMA

#### 3.1. Chapter overview and key findings

In this chapter, I have performed a detailed characterisation of pathological hallmarks of the *Smn*<sup>2B/-</sup> mouse model in order to determine the pattern of selective vulnerability that is present in this model. I have performed a thorough body wide analysis of neuromuscular junction pathology, coupled with a quantification of the loss of motor neurons at different spinal cord levels. A distinct pattern of selective vulnerability was revealed in the *Smn*<sup>2B/-</sup> mouse model. I have further compared this distinct pattern of selective vulnerability to the vulnerability patterns of three other commonly used mouse models of SMA to determine their similarities and differences.

The principal findings of the following chapter are:

- Muscles innervated by cranial nerves and lower lumbar spinal nerves show little NMJ pathology in the *Smn*<sup>2B/-</sup> mouse model of SMA.
- There are higher levels of synaptic loss in the core and proximal limbs compared to the distal limbs and head in the *Smn*<sup>2B/-</sup> mouse model of SMA.
- Regional NMJ pathology correlates with motor neuron cell body loss in the spinal cord of the *Smn*<sup>2B/-</sup> mouse model of SMA.
- The regional pattern of selective vulnerability in the *Smn*<sup>2B/-</sup> mouse model shows remarkable similarity to the vulnerability pattern observed in SMA patients.
- Distinct patterns of selective vulnerability are present in the four most commonly utilised mouse models of SMA: the *Smn*<sup>-/-</sup>;SMN2, Taiwanese, SMNΔ7 and *Smn*<sup>2B/-</sup> mouse models.
- Although mouse models of SMA show drastic differences in patterns of selective vulnerability, each model shows a similar range of synaptic loss.

### 3.2. Introduction

In SMA, the NMJs present early and important pathological targets of the disease (Murray et al., 2008). Previous work reported a wide range of morphological and functional defects of NMJs in mouse models of SMA (Dachs et al., 2011; Kariya et al., 2008; Kong et al., 2009; Ling et al., 2012; Murray et al., 2008). Further, it was shown that NMJ loss coincides with the onset of cell death pathways at the motor neuron cell body, but that it precedes a quantifiable loss of motor neurons (Buettner et al., 2021; Courtney et al., 2019). The sequential breakdown of motor neurons is relatively well defined in SMA, however not all motor neurons are uniformly vulnerable. Some motor neurons are lost early during the course of the disease, while others remain intact at late stages of the disease. This selective vulnerability of motor neurons has long been recognised in SMA patients. It has been documented that core, proximal arm and proximal leg muscles show more profound weakness than muscles of the face and distal limbs in SMA patients (Deymeer et al., 2008; Dubowitz, 1999; Wadman et al., 2018).

The reasons for selective vulnerability of motor neurons and their associated muscles in SMA are currently unclear, but it is becoming increasingly important to understand the underlying patterns of selective vulnerability and investigate their basis. A lot of research of selectively vulnerable muscles has been done on commonly utilised mouse models of SMA: the *Smn*<sup>-/-</sup>; *SMN2*, the Taiwanese, and the *SMN*Δ7 mouse model. A wide range of muscles has been examined across these different mouse models by analysing and classifying their synaptic loss, showing a high variability in levels of NMJ loss between muscles (Buettner et al., 2021; Comley et al., 2016; Lin et al., 2016; Ling et al., 2012; Murray et al., 2010a, 2008; Thomson et al., 2012). Previous work on the severe *Smn*<sup>-/-</sup>; *SMN2* mouse model investigated a number of head, thoracic, abdominal and distal hindlimb muscles at disease end-stage P5-P6 (Murray et al., 2010a, 2008; Thomson et al., 2012). A comprehensive study in the Taiwanese mouse model investigated the levels of NMJ loss in 25 axial and appendicular muscles at disease end-stage P9 (Lin et al., 2016). Additionally, muscles of the back and distal hindlimbs were investigated in this model (Buettner et al., 2021). To date, the most characterisations of selectively

vulnerable muscles have been performed in the SMN $\Delta$ 7 mouse model. A comprehensive study investigated 23 axial and appendicular muscles at disease end-stage P12-P14 in this model, including various head, neck, back, thoracic, proximal forelimb, and proximal and distal hindlimb muscles (Ling et al., 2012). Several other studies investigated further head, thoracic, abdominal, back, forelimb and hindlimb muscles in the SMN $\Delta$ 7 mouse model (Buettner et al., 2021; Comley et al., 2016; Murray et al., 2008).

Additionally, the more recently developed less severe *Smn*<sup>2B/-</sup> mouse model has become a commonly utilised model of the disease. In contrast to the other SMA mouse model, only a small number of muscles has previously been investigated for selective vulnerability in the *Smn*<sup>2B/-</sup> model (Buettner et al., 2021; Murray et al., 2015, 2013). To date, only some head and abdominal muscles (Courtney et al., 2019; Murray et al., 2015, 2013) as well as one back and distal hindlimb muscle have been characterised (Buettner et al., 2021). The comparatively long lifespan of the *Smn*<sup>2B/-</sup> mouse model and the late symptomatic onset (P10) offer an extended pre-symptomatic time window that cannot be seen in other mouse models of SMA. This prolonged pre-symptomatic window facilitates the study of the temporal and molecular pathological events that ultimately result in motor neuron death in this model. The *Smn*<sup>2B/-</sup> model poses a valuable system for an array of different investigations. This includes time-course analysis throughout different symptomatic stages and an extended timeframe for investigation of regenerative processes following treatment. It is therefore important to conduct a comprehensive body wide analysis of selectively vulnerable muscles in the *Smn*<sup>2B/-</sup> model, to gain a better understanding of the underlying NMJ and motor neuron pathology present in this model.

Although there have been various publications investigating NMJ pathology and synaptic loss in the above-mentioned mouse models of SMA, there has not yet been a comprehensive comparison of the resulting patterns of selective vulnerability in these models. A direct comparison of the consistency of vulnerability patterns, including their similarities and differences between models, can help to understand and identify conserved mechanisms and

temporal series of degenerative and developmental events that underlie motor unit pathology. The value of such a comparative study was also recently demonstrated on a smaller scale by comparing some individual muscles across three different mouse models of SMA, thereby providing information to better tailor future studies (Buettner et al., 2021). By comparing vulnerability patterns across different SMA mouse models, we can learn how to utilise this newly generated knowledge of differential vulnerability. The similarities and differences in selective vulnerability patterns across mouse models of SMA can be utilised as tool to make informed decisions about tailored experimental designs in future studies and can provide a new platform to uncover the mechanism driving motor neuron resistance and susceptibility.

In this chapter of this thesis, I aimed to perform a detailed characterisation of the NMJ pathology present in the *Smn*<sup>2B/-</sup> mouse model, and to compare patterns of selective vulnerability across the four most commonly utilised mouse models of SMA. In this body of work, I performed a thorough body wide analysis of the levels of synaptic loss in 23 muscles of the *Smn*<sup>2B/-</sup> mouse model of SMA. I showed that this mouse model displays a unique regional pattern of selective vulnerability, where muscles of the core, proximal forelimb and proximal hindlimb demonstrated higher levels of NMJ loss than muscles of the head, distal forelimb, distal hindlimb and foot muscles. The increased vulnerability of muscles innervated by the centre of the spinal cord correlates with loss of motor neuron cell bodies in the associated spinal cord segments. I subsequently assembled this data combined with previously published results from analyses of selectively vulnerable muscles in the *Smn*<sup>-/-</sup>;SMN2, Taiwanese, SMNΔ7 and *Smn*<sup>2B/-</sup> mouse models and used a seven-point colour-coded classification tool to compare patterns of selective vulnerability between SMA mouse models. I found that while each mouse model of SMA displayed the same range of synaptic loss (30-100%), each model displayed a unique and distinct pattern of selective vulnerability. Overall, data presented in this chapter demonstrates that there are drastic differences between selective vulnerability pattern across mouse models of SMA, and that these differences need to be considered in the design of future studies.

### 3.3. Results

#### 3.3.1. A regional pattern of neuromuscular junction pathology is present in the *Smn*<sup>2B/-</sup> mouse model

In this chapter, I performed a thorough body wide analysis of NMJ and motor neuron pathology of the *Smn*<sup>2B/-</sup> mouse model of SMA in order to generate a detailed characterisation of the selective vulnerability in this model. In our hands, the *Smn*<sup>2B/-</sup> mouse model displays a motor phenotype from around P10 and has a life expectancy of around 18 days. I aimed to comprehensively assess the level of NMJ pathology present in each investigated muscle of the *Smn*<sup>2B/-</sup> mouse model. For this analysis, the NMJ pathology was analysed by quantification of the occupancy of endplates from P16 mice. Occupancy measures the level of innervation of the motor endplate and therefore determines the level of denervation within a muscle. A full description of the criteria of occupancy analysis in immuno-stained muscles can be found in Chapter 2 section 2.5.2. Briefly, endplates are fully innervated if they are completely covered by the pre-synaptic terminal, partially occupied if they are only partly covered by the pre-synaptic terminal, and vacant if there is no innervation of the endplate left (Chapter 2, Figure 2.3A). NMJ pathology was assessed in 23 muscles from nine different body regions: head muscles, neck muscles, thoracic muscles, abdominal muscles, proximal and distal forelimb muscles, proximal and distal hindlimb muscles, and foot muscles.

NMJ pathology was assessed in four head and neck muscles (Figure 3.1). The digastric posterior (DP) and masseter (MA), two muscles of the head, showed no significant increase in vacant endplates, but displayed a significantly reduced amount of fully innervated endplates with a mean of 91.8% and 89.3% innervated motor endplates respectively (Figure 3.1D-E). In contrast, investigated neck muscles not only showed a significant reduction in fully innervated endplates, with only 29.3% innervation remaining in the sternocleidomastoid (SCM) and 73.4% innervation in the sternohyoid (SH), but they also displayed a significant increase in vacant motor endplates (Figure 3.1F-G). This showed that the neck muscles, especially the SCM, were prone to vulnerability, whilst the head muscles showed only mild levels of denervation (maximum of 10.7%) in the *Smn*<sup>2B/-</sup> mouse model.

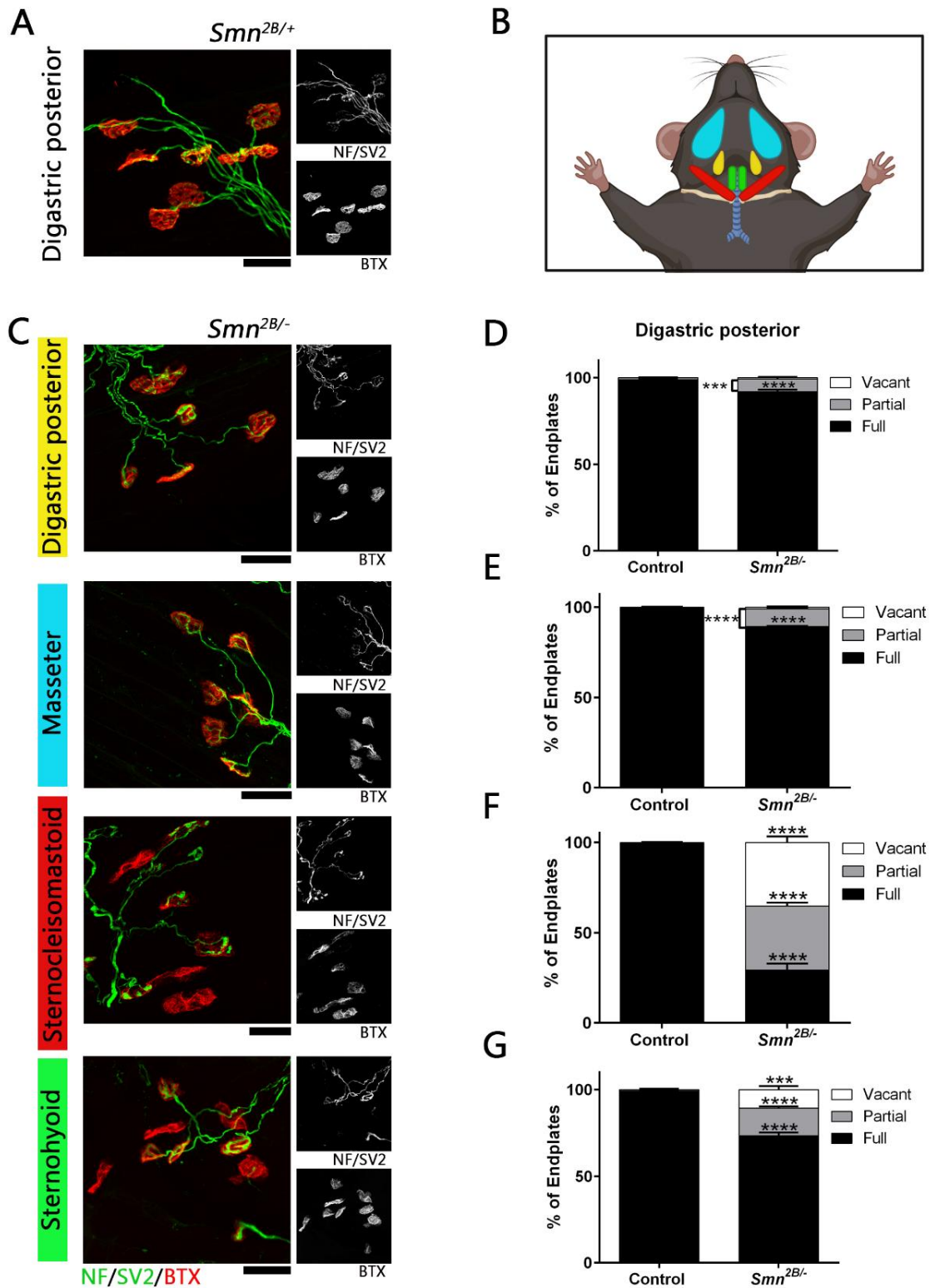


**Figure 3.1: A significant amount of synaptic loss is observed in the neck muscles, whereas head muscles show no significant amount of synaptic loss in the *Smn*<sup>2B/-</sup> mouse model at P16. (A)** Representative confocal image showing NMJs with its pre-synaptic terminal labelled with antibodies against neurofilament (NF, 2H3; green) and synaptic vesicle protein 2 (SV2; green) and the motor endplates labelled with  $\alpha$ -Bungarotoxin (BTX; red) from a control digastric posterior (DP). Scale bar: 25 $\mu$ m. **(B)** Schematic diagram of the anatomical locations of head and neck muscles (digastric posterior [DP], masseter [MA], sternocleidomastoid [SCM] and sternohyoid [SH]) in a mouse in supine position. **(C)** Representative confocal images showing NMJs labelled with antibodies against NF (green),

SV2 (green) and BTX (red) from DP, MA, SCM and SH. Scale bar: 25µm. **(D-G)** Bar charts showing the quantification of percentage of full, partial and vacant endplates in *Smn*<sup>2B/-</sup> mice compared to controls in DP, MA, SCM and SH respectively. Note that the neck muscles (SCM and SH) show a significant increase in vacant motor endplates and a significant decrease in fully occupied endplates, while the head muscles (DP and MA) do not show a significant increase in vacant motor endplates. Two-sided ANOVA with Sidak correction (\*\*p≤0.001 and \*\*\*p≤0.0001). n= 3,4 in all muscles for control and *Smn*<sup>2B/-</sup> mice respectively. 3 FOV quantified directly under the microscope for one muscle each per n-number. Error bars represent mean ± SEM.

I further investigated synaptic loss in muscles of the thorax and abdomen (Figure 3.2). The intercostals (IC) and triangularis sterni (TS) were analysed as thoracic muscles and displayed a significant increase in vacant motor endplates, and a significant decrease in the percentage of fully innervated endplates with 58.1% and 73.0% innervation respectively (Figure 3.2D-E). The same pattern was visible upon investigation of three abdominal muscles, the external oblique (EO), rectus abdominis (RA) and the transversus abdominis muscle (TVA). All three abdominal muscles showed both, a significant increase in vacant and a significant decrease in fully occupied endplates (Figure 3.2F-H). The RA showed the most vulnerability with 56.2% innervation remaining, compared to the EO with 76.5% and the TVA with 67.4% of fully innervated endplates. The TVA muscle was previously proven to display a similar amount of NMJ loss at disease end-stage (Courtney et al., 2019; Murray et al., 2015), hereby validating the presented data. Taken together, the data from the thoracic and abdominal muscles indicates that muscles from both body regions seem to be vulnerable in the *Smn*<sup>2B/-</sup> mouse model.

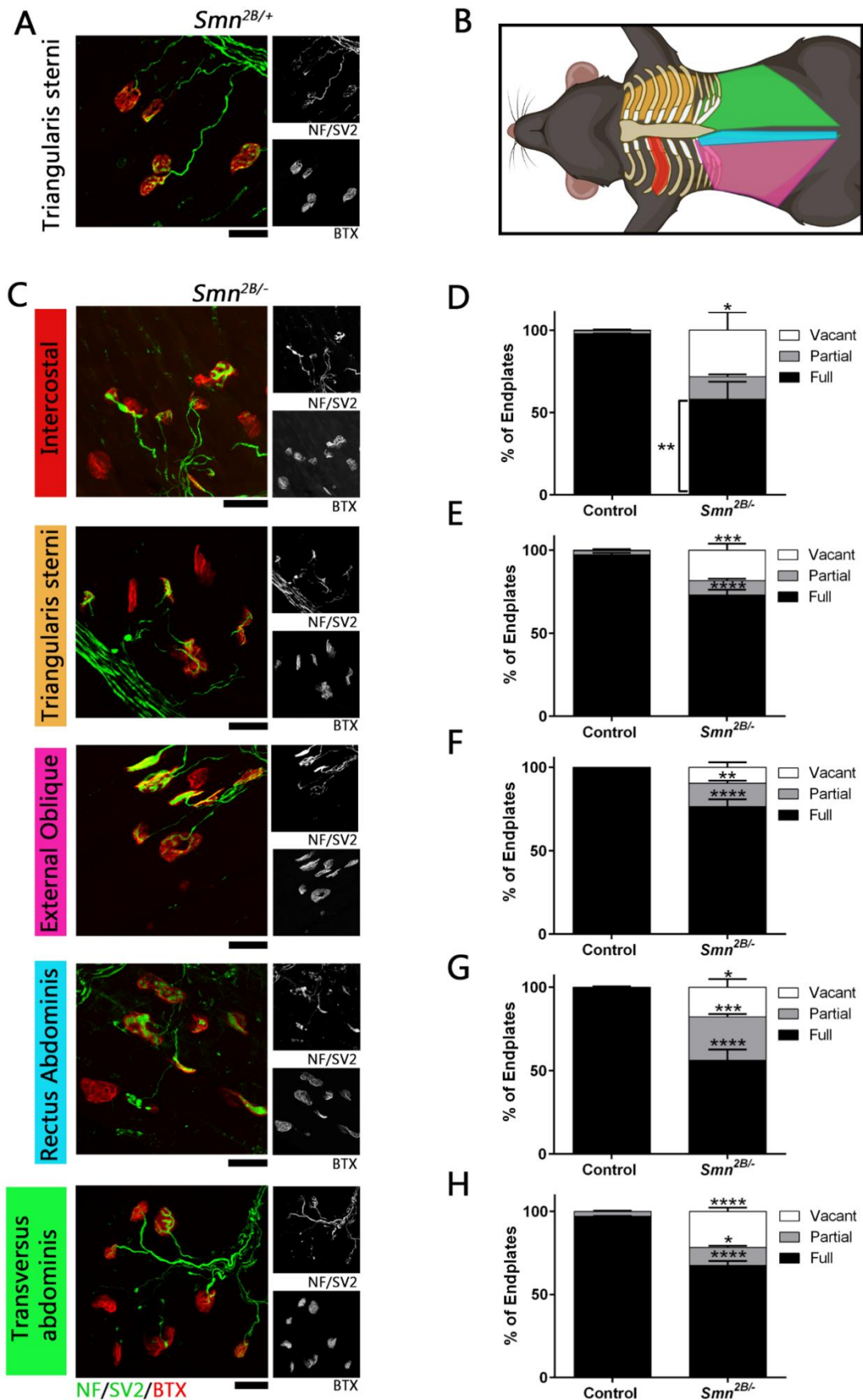


**Figure 3.2: A significant amount of synaptic loss is observed in the thoracoabdominal muscles of the *Smn*<sup>2B/-</sup> mouse model of SMA at P16. (A) Representative confocal image showing NMJs with its pre-synaptic terminal labelled with antibodies against NF and SV2**

(green) and the motor endplates labelled with BTX (red) from a control triangularis sterni (TS). Scale bar: 25µm. **(B)** Schematic diagram of the anatomical locations of thoracic muscles (intercostals [IC] and triangularis sterni [TS]) and abdominal muscles (external oblique [EO], rectus abdominis [RA] and transversus abdominis [TVA]) in a mouse in supine position. **(C)** Representative confocal images showing NMJs labelled with antibodies against NF (green), SV2 (green) and BTX (red) from IC, TS, EO, RA and TVA. Scale bar: 25µm. **(D-H)** Bar charts showing the quantification of percentage of full, partial and vacant endplates in *Smn*<sup>2B/-</sup> mice compared to controls in IC, TS, EO, RA and TVA respectively. Note that all thoracoabdominal muscles show a significant increase in vacant motor endplates and a significant decrease in fully occupied endplates. Two-sided ANOVA with Sidak correction (\*p≤0.05, \*\*p≤0.01, \*\*\*p≤0.001 and \*\*\*\*p≤0.0001). n=3 in IC for controls and *Smn*<sup>2B/-</sup> mice; n=3,4 in all other muscles for controls and *Smn*<sup>2B/-</sup> mice respectively. 3 FOV quantified directly under the microscope for one muscle each per n-number. Error bars represent mean ± SEM.

Subsequently, NMJ pathology in muscles from the proximal and distal forelimb was analysed (Figure 3.3). The proximal forelimb muscles biceps brachii (BC), deltoid (DE) and triceps brachii (TC) all displayed severe NMJ loss with only 56.0%, 71.3% and 34.4% remaining fully innervated endplates respectively (Figure 3.3D-F). A significant decrease in fully innervated endplates was further shown in the flexor carpi radialis (FCR) of the distal forelimb, with 79.6% remaining fully occupied motor endplates (Figure 3.3G). Additionally, a significant increase in vacant endplates was shown in all investigated proximal and distal forelimb muscles. This data shows that the proximal forelimb seems to be more severely affected by NMJ pathology than the distal forelimb. Despite differences in vulnerability levels between investigated proximal and distal forelimb muscles, all investigated forelimb muscles seem to be vulnerable muscles, by showing significantly increased levels of vacant endplates in the *Smn*<sup>2B/-</sup> mouse model.

Lastly, muscles from the proximal and distal hindlimb, and foot muscles were analysed (Figure 3.4). Quantification of the quadriceps (QC) and gracilis (GR) revealed a significant increase in vacant endplates and a significant decrease in their amount of fully innervated endplates, with 64.8% in the QC and 62.0% innervation remaining in the GR (Figure 3.4F). In contrast to the proximal hindlimb muscles, quantification of the distal hindlimb muscles extensor digitorum longus (EDL), gastrocnemius (GS), soleus (SO) and the tibialis anterior (TA) revealed that none of these muscles showed a significant amount of vacant endplates, and only the GS and the TA showed a slight reduction in

their number of fully occupied endplates with 84.8% and 88.5% respectively (Figure 3.4G). The EDL and SO did not show any significant difference in their numbers of fully and partially innervated or vacant endplates. Further, the quantification of synaptic loss in the plantar foot muscles flexor digitorum brevis, with its heads on digits 2, 3 and 4 (FDB2, FDB3 and FDB4), and the lumbricals (LU) were analysed (Figure 3.4H). These muscles revealed a similar pattern to the distal hindlimb muscles. None of the investigated foot muscles displayed an increase in vacant endplates. The LU remained almost fully innervated with 98.94% innervation remaining, and the three heads of the FDB only lost minor amounts of NMJs with 86.2%, 86.0% and 84.9% fully innervated endplates remaining in the FDB2, FDB3 and FDB4 respectively. Taking these results together, the hindlimb muscles display a high variability of vulnerability. While the proximal hindlimb muscles show significantly increased levels of vacant endplates, the distal hindlimb muscles and the foot muscles do not show significant levels of denervated endplates at late stages of the disease, suggesting the distal hindlimb and foot muscles are resistant in this mouse model of SMA.

The presented results of this comprehensive analysis of NMJ pathology across nine different body regions in the *Smn*<sup>2B/-</sup> mouse model of SMA showed that there seems to be a complex pattern of selective vulnerability present. Each of the nine investigated body regions seems to be solely comprised of muscles that either all show significant amounts of denervation, or muscles that all show no significant amount of fully denervated endplates in this SMA mouse model.

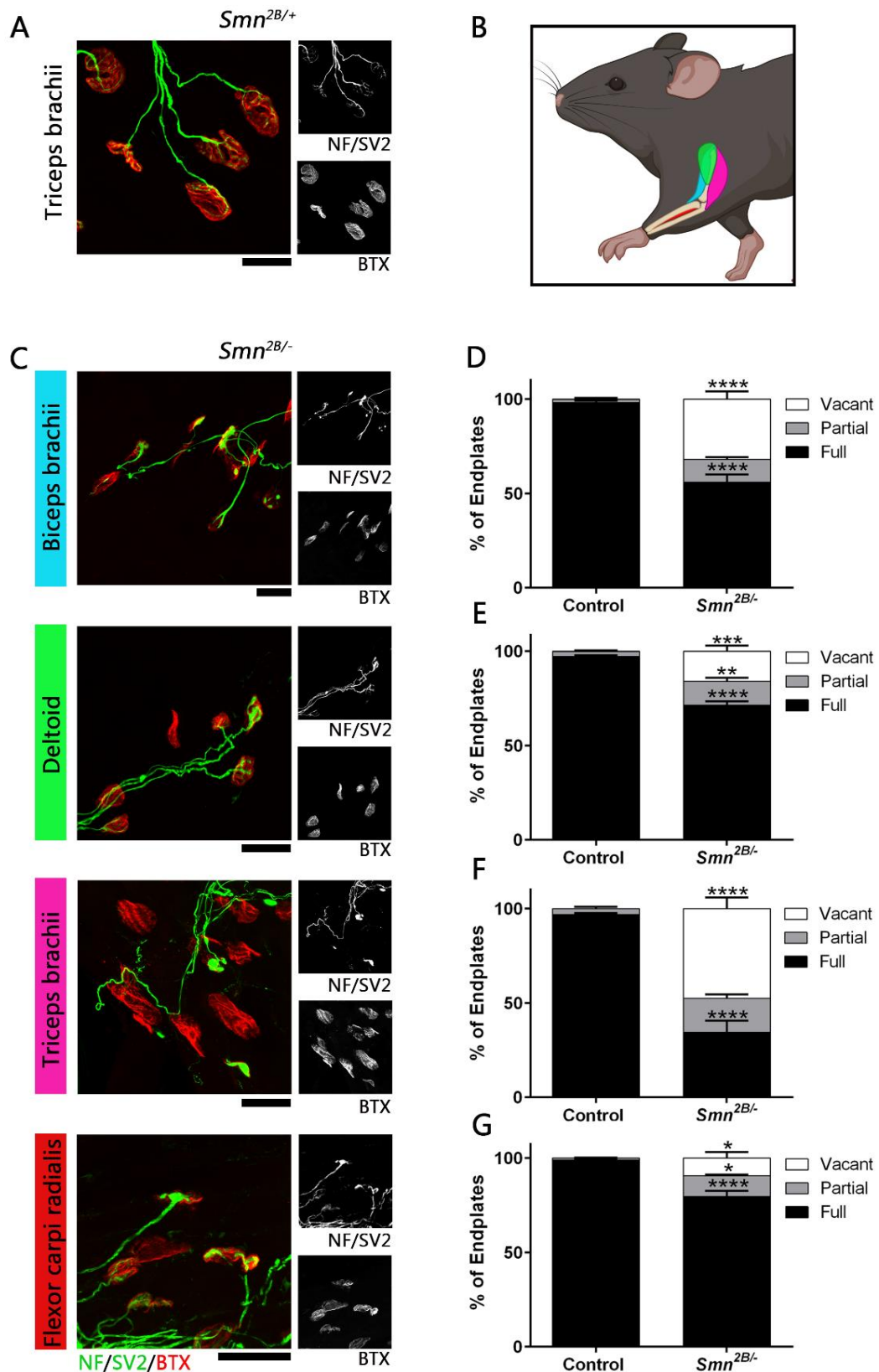


**Figure 3.3: A significant amount of synaptic loss is observed in proximal and distal forelimb muscles in the *Smn*<sup>2B/-</sup> mouse model at P16. (A)** Representative confocal image showing NMJs with its pre-synaptic terminal labelled with antibodies against NF and SV2 (green) and the motor endplates labelled with BTX (red) from a control biceps brachii (BC). Scale bar: 25µm. **(B)** Schematic diagram of the anatomical locations of proximal forelimb muscles (biceps brachii [BC], deltoid [DE] and triceps brachii [TC]) and distal forelimb muscle

(flexor carpi radialis [FCR]) in a laterally viewed mouse. **(C)** Representative confocal images showing NMJs labelled with antibodies against NF (green), SV2 (green) and BTX (red) from BC, DE, TC and FCR. Scale bar: 25µm. **(D-G)** Bar charts showing the quantification of percentage of full, partial and vacant endplates in *Smn*<sup>2B/-</sup> mice compared to controls in BC, DE, TC and FCR respectively. Note that all proximal and distal forelimb muscles show a significant increase in vacant motor endplates and a significant decrease in fully occupied endplates. Two-sided ANOVA with Sidak correction (\*p≤0.05, \*\*p≤0.01, \*\*\*p≤0.001 and \*\*\*\*p≤0.0001). n=3,4 for controls and *Smn*<sup>2B/-</sup> mice respectively. 3 FOV quantified directly under the microscope for one muscle each per n-number. Error bars represent mean ± SEM.

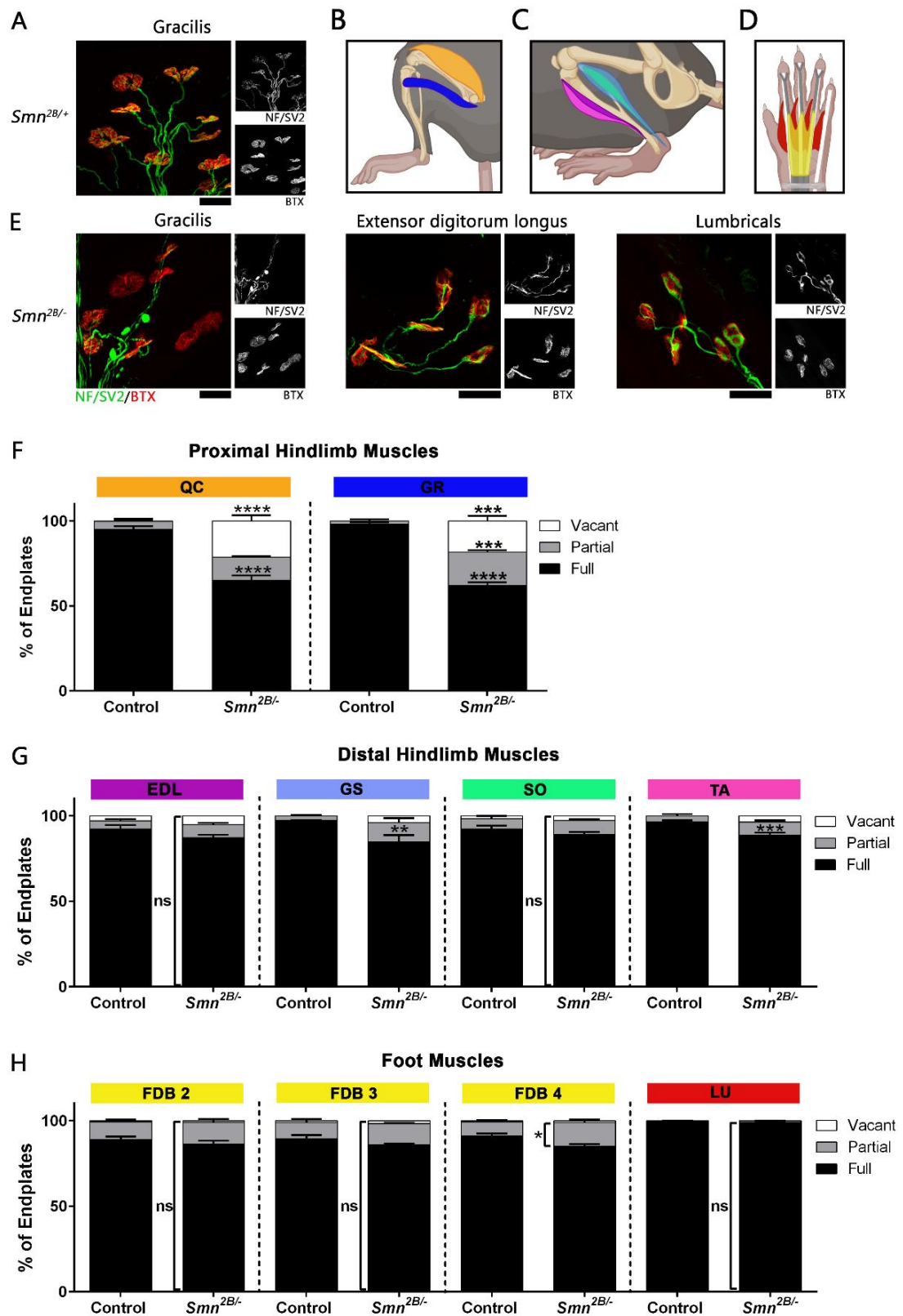


**Figure 3.4: A significant amount of synaptic loss is observed in proximal hindlimb muscles, whereas distal hindlimb and foot muscles show no significant amount of synaptic loss in the *Smn*<sup>2B/-</sup> mouse model at P16. (A) Representative confocal image showing NMJs with its pre-synaptic terminal labelled with antibodies against NF and SV2 (green) and the motor endplates labelled with BTX (red) from a control gracilis (GR). Scale bar: 25µm. (B) Schematic diagram of the anatomical locations of proximal hindlimb muscles (quadriceps [QC] and gracilis [GR]) in a mouse in supine position. (C) Schematic diagram of**

the anatomical locations of distal hindlimb muscles (extensor digitorum longus [EDL], gastrocnemius [GS], soleus [SO] and tibialis anterior [TA]) in a laterally viewed mouse. **(D)** Schematic diagram of the anatomical locations of foot muscles (flexor digitorum brevis 2, 3 and 4 [FDB 2, FDB 3, FDB 4] and lumbrical [LU]) in a plantar viewed hindlimb paw. **(E)** Representative confocal images showing NMJs labelled with antibodies against NF (green), SV2 (green) and BTX (red) from GR, EDL and LU. Scale bar: 25µm. **(F-H)** Bar charts showing the quantification of percentage of full, partial and vacant endplates in *Smn*<sup>2B/-</sup> mice compared to controls in proximal hindlimb muscles (QC and GR), distal hindlimb muscles (EDL, GS, SO and TA), and foot muscles (FDB 2, FDB 3, FDB 4 and LU) respectively. Note that all proximal hindlimb muscles show a significant increase in vacant motor endplates and a significant decrease in fully occupied endplates, whereas none of the distal hindlimb and foot muscles show a significant increase in vacant endplates. Two-sided ANOVA with Sidak correction (ns= no significance, \*p≤0.05, \*\*p≤0.01, \*\*\*p≤0.001 and \*\*\*\*p≤0.0001). n=3,3 in GR for controls and *Smn*<sup>2B/-</sup> mice; n=3,4 in all other muscles for controls and *Smn*<sup>2B/-</sup> mice respectively. 3 FOV quantified directly under the microscope for one muscle each per n-number. Error bars represent mean ± SEM.

### **3.3.2. Higher levels of neuromuscular junction loss are present at cervical, thoracic, and upper lumbar levels of the spinal cord in the *Smn*<sup>2B/-</sup> mouse model**

I investigated whether there is a correlation between relative vulnerability of muscles and spinal innervation level. All investigated muscles from *Smn*<sup>2B/-</sup> animals were arranged by their level of innervation from the spinal cord (Figure 3.5). Head muscles (MA and DP) innervated by cranial nerve CN VII seemed to be comparatively resistant to degeneration. This is consistent with published work showing a sparing of cranial muscles that are also innervated by CN VII in the *Smn*<sup>2B/-</sup> mouse model (Murray et al., 2015, 2013). A higher amount of vulnerability was observed in the neck muscles (SCM and SH) and forelimb muscles (DE, BC, TC and FCR) which are innervated by the cervical spinal nerves. There was also an increased vulnerability in muscles that are innervated by the thoracic spinal nerves, namely in the thoracic muscles (IC and TS) and the abdominal muscles (EO, RA and TVA). A similar level of synaptic loss was also seen in the proximal leg muscles (GR and QC), which are innervated by the upper lumbar spinal nerves. Contrary to the proximal hindlimb muscles, the distal hindlimb muscles (TA, EDL, GS and SO) and foot muscles (FDB2, FDB3, FDB4 and LU), innervated by the middle and lower lumbar spinal nerves, did not show a large amount of synaptic loss. These observations taken together, demonstrate that the *Smn*<sup>2B/-</sup> mouse model of SMA shows a regional pattern of vulnerability. Increased vulnerability was found in muscles that are innervated by cervical, thoracic, and upper lumbar

spinal nerves. These regions are more prone to vulnerability than muscles that are innervated by cranial nerves and the middle and lower lumbar spinal nerves. These findings show a pattern of vulnerability consistent with increased vulnerability in the muscles of the core, neck and proximal limbs with lower levels of NMJ pathology in the muscles of the distal limbs and face.

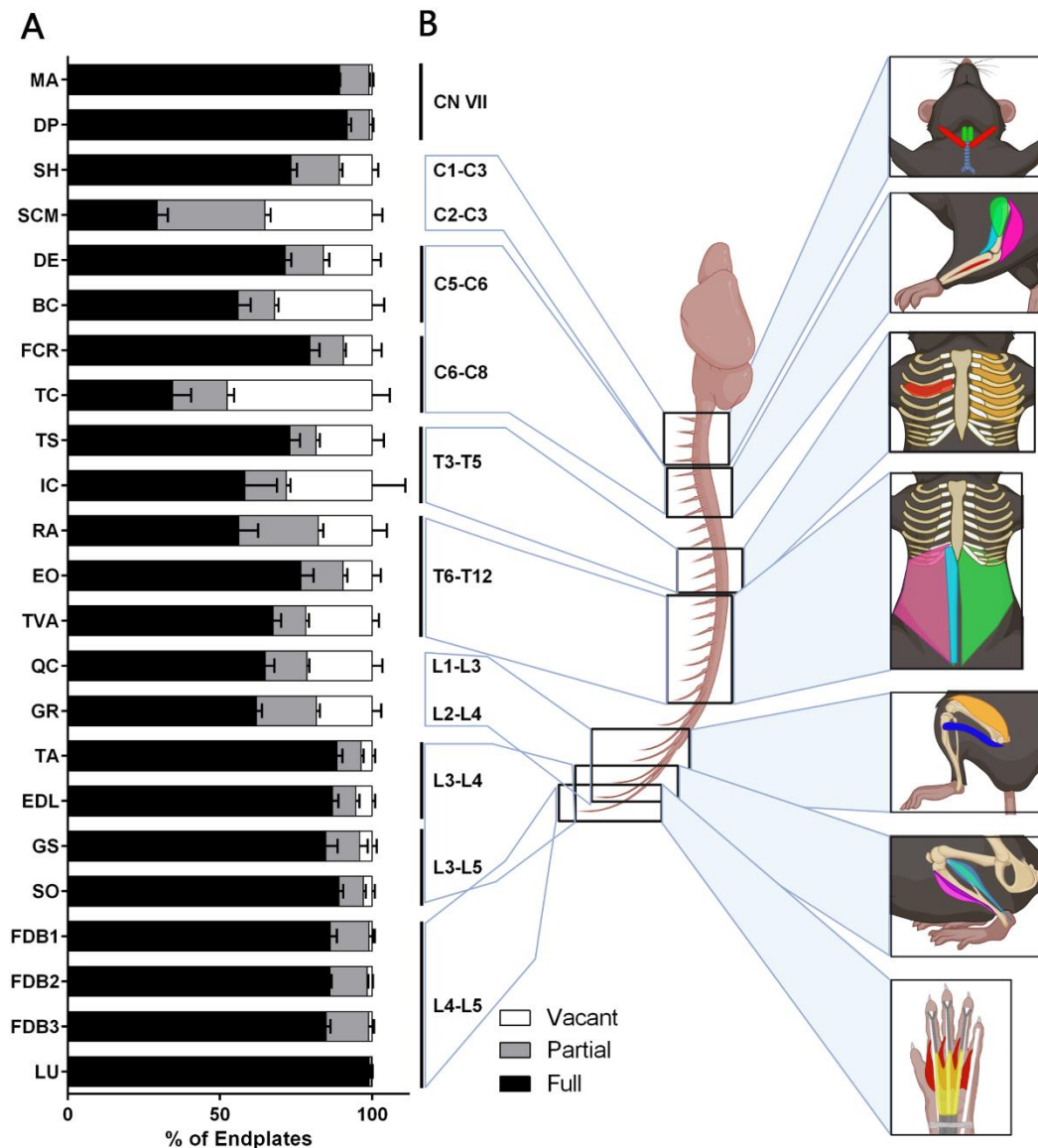


**Figure 3.5: NMJ loss is most profound in muscles innervated by spinal nerves from lower cervical, thoracic, and upper lumbar spinal cord segments in the *Smn*<sup>2B/-</sup> mouse model at P16. (A-B)** Bar charts showing the quantification of percentage of full, partial and vacant endplates in *Smn*<sup>2B/-</sup> mice in all investigated muscles. Muscles are displayed in order of their innervation from cranial nerves and spinal cord segments, starting at the top with muscles innervated by cranial nerve VII (CN VII), followed by muscles innervated from nerves arising from the cervical spinal cord and ending at the bottom with muscles innervated by nerves arising from the lumbar spinal cord. The spinal cord segments that supply nerve innervation to the analysed muscles are indicated beside the respective bars. An increase in

NMJ loss is visible across muscles that are innervated by nerves across cervical, thoracic, and upper lumbar spinal cord segments. This pattern suggests that NMJ pathology starts at the centre of the spinal cord.  $n=3$  in GR, IC for *Smn*<sup>2B/-</sup> mice;  $n=4$  in all other muscles for *Smn*<sup>2B/-</sup> mice. 3 FOV quantified directly under the microscope for one muscle each per  $n$ -number. Error bars represent mean  $\pm$  SEM. **(B)** Schematic diagram of the relationship between location of spinal cord segments and the anatomical location of muscles innervated by nerves arising from the indicated spinal cord segments, starting with spinal nerves C1-C3.

### **3.3.3. Regional neuromuscular junction pathology correlates with motor neuron cell body loss in the spinal cord of the *Smn*<sup>2B/-</sup> mouse model**

To investigate if motor neuron loss indeed correlates with increased vulnerability of muscles innervated by the corresponding spinal cord segments, I quantified the motor neuron cell body numbers in the ventral horn of three different spinal cord segments from thoracic and lumbar regions (see Chapter 2, section 2.5.5.). Middle and lower thoracic spinal segments T5 and T11, and lower lumbar spinal cord segments L5 were quantified from *Smn*<sup>2B/-</sup> and control mice at disease end-stage P16 (Figure 3.6). I observed a significant loss of motor neuron cell bodies ( $p \leq 0.05$ ) in spinal cord segments T5 and T11 in *Smn*<sup>2B/-</sup> compared to their controls (Figure 3.6B-C). In contrast to the investigated thoracic regions, there was no significant difference in the total number of motor neuron cell bodies present in the lower lumbar spinal cord segment L5 between *Smn*<sup>2B/-</sup> and control mice at P16 (Figure 3.6D). These findings indicate that synaptic loss is indeed associated with the loss of motor neurons. These results provide further evidence that there is an increased vulnerability in the thoracic region compared to lower lumbar levels in the *Smn*<sup>2B/-</sup> mouse model of SMA.

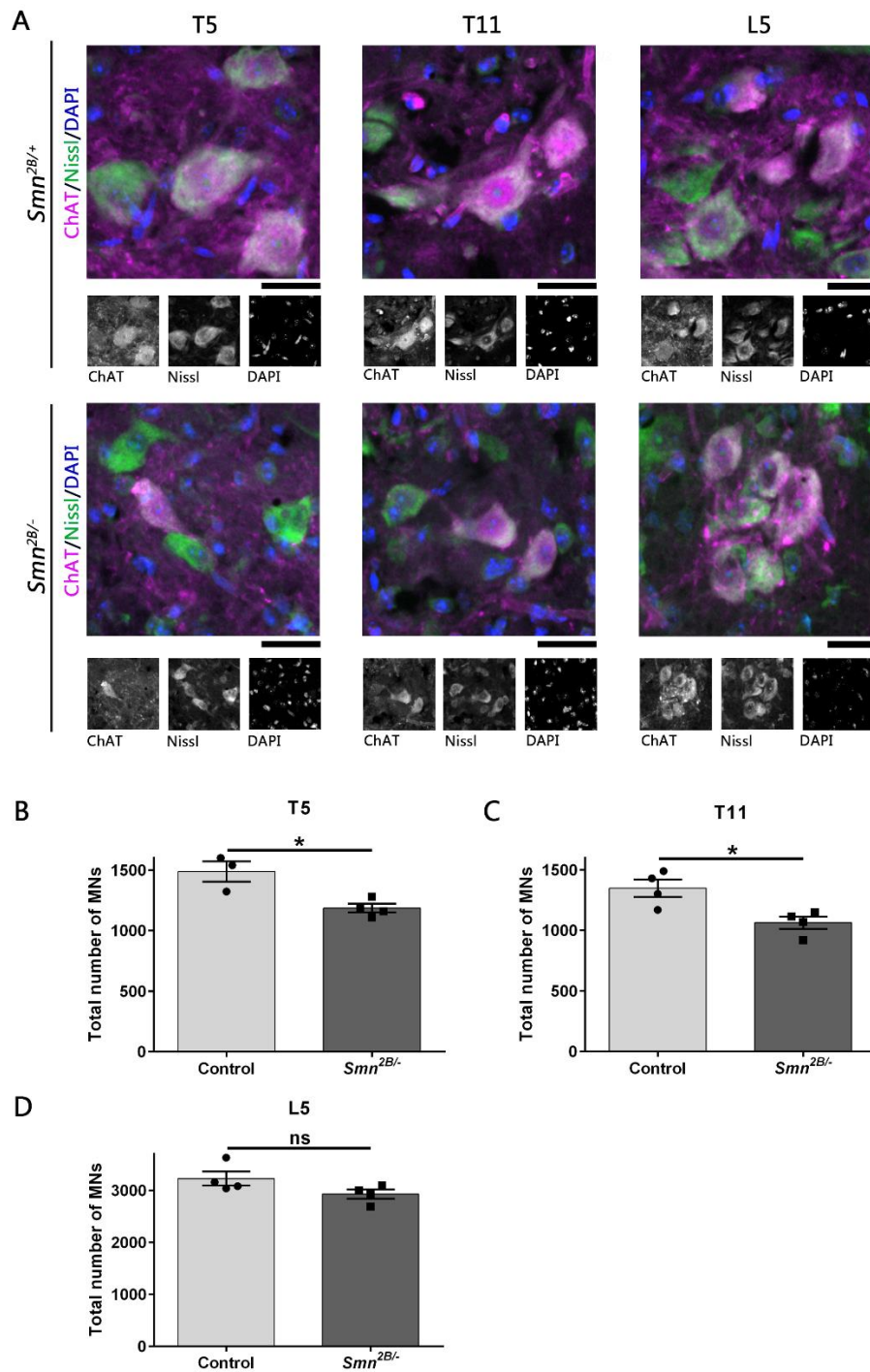


**Figure 3.6: A significant decrease in the total number of motor neurons is observed in thoracic spinal cord segments in the *Smn*<sup>2B/-</sup> mouse model at P16. (A)** Representative images showing motor neurons labelled with antibodies against anti-choline acetyltransferase (ChAT; red), Nissl (green) and DAPI (blue) from *Smn*<sup>2B/-</sup> and control mice in thoracic spinal cord segments (T5 and T11) and lumbar spinal cord segment L5 at P16. Scale bar: 50µm. **(B-D)** Bar charts showing the total number of motor neurons quantified in the ventral horn in the spinal cord segments T5, T11 and L5 in *Smn*<sup>2B/-</sup> mice compared to their controls. There is a significant decrease in the total number of counted motor neurons in *Smn*<sup>2B/-</sup> mice in both thoracic spinal cord segments. However, there is no significant difference in the total number of motor neurons between genotypes in the lumbar segment. Unpaired student's t-test (ns= no significance, \*p≤0.05). n=3,4 for controls and *Smn*<sup>2B/-</sup> mice respectively in T5; n=4 for both genotypes in all other spinal cord segments. For each spinal cord segment, one FOV was quantified directly under the microscope on 9 sections per n-number to calculate total motor neuron count for each sample. Error bars represent mean ± SEM.

### 3.3.4. Comparison of neuromuscular junction pathology reveals similar range of synaptic loss across four mouse models of SMA

Several previous studies have utilised four available mouse models of SMA to classify the relative vulnerability of a variety of muscles in these SMA mouse models (Buettner et al., 2021; Comley et al., 2016; Courtney et al., 2019; Lin et al., 2016; Ling et al., 2012; Murray et al., 2015, 2013, 2010a, 2008; Thomson et al., 2012). There have been various publications on selectively vulnerable muscles from the *Smn*<sup>-/-</sup>;SMN2, the Taiwanese and the SMNΔ7 mouse models of SMA, providing a detailed characterisation of these mouse models. However, to date there has not yet been an overview and comprehensive comparison of the resulting patterns of selective vulnerability in these commonly utilised mouse models of SMA. In order to gain insight into the consistency of vulnerability patterns across mouse models of SMA, I compared patterns of selectively vulnerable muscles across the four most commonly utilised models. I compared previously published data from the *Smn*<sup>-/-</sup>;SMN2, Taiwanese, SMNΔ7 and *Smn*<sup>2B/-</sup> mouse model, and supplemented it with my own analysis from the *Smn*<sup>2B/-</sup> mouse model.

Despite the large amount of previously characterised muscles in the SMNΔ7 mouse model, there was only limited data available on cranial muscles and muscles from the thorax and abdomen in this model. Since in the *Smn*<sup>2B/-</sup> mouse model the cranial muscles proved to be resistant to degeneration, while both thoracic and abdominal muscles proved to show high levels of vulnerability, it was important to be able to compare the vulnerability pattern present in these body regions to other mouse models of SMA. I supplemented the obtained SMNΔ7 data with an additional analysis of five muscles from the above mentioned three different body regions in this mouse model (Figure 3.7). I quantified the synaptic loss in two cranial muscles (AAL and AS), a thoracic muscle (TS), and two abdominal muscles (EO and RA) in the SMNΔ7 mouse model of SMA at time point P12 at disease end-stage (Figure 3.7). Quantification of cranial muscles showed that there was a significant decrease in fully innervated endplates with 53.1% and only 14.7% motor endplates remaining in the AAL and AS muscles respectively (Figure 3.7D). Additionally, a significant increase in vacant endplates could be observed in

the AS muscle. Both cranial muscles show high levels of vulnerability in the SMN $\Delta$ 7 mouse model. In contrast to the highly vulnerable cranial muscles, the thoracic muscle TS and the abdominal muscles EO and RA all showed only mild reductions in fully innervated endplates, with 86.1%, 88.7% and 79.0% endplates remaining respectively (Figure 3.7E-F). None of the thoracic and abdominal muscles showed a significant increase in vacant endplates. These muscles only seem to be mildly affected at disease end-stage.

Following the analysis of these additional muscles from the SMN $\Delta$ 7 mouse model, I now had obtained data sets of previously published data from the *Smn*<sup>-/-</sup>;SMN2, Taiwanese, SMN $\Delta$ 7 and *Smn*<sup>2B/-</sup> mouse model, supplemented with my own analysis from the *Smn*<sup>2B/-</sup> and SMN $\Delta$ 7 mouse models. These data sets were utilised to gain insight into the consistency of vulnerability patterns across the four most commonly used mouse models of SMA. Towards this goal, I developed a seven-point colour-coded classification tool for vulnerability. All muscles were classified based upon the percentage of remaining fully innervated motor endplates at disease end stage in the given model. Each category was given a specific colour from dark green to dark magenta to represent a 10% range of remaining fully innervated endplates. For example, the dark green category represents muscles with 100-91% fully innervated endplates and the light green category represents muscles with 90-81% (see legend in Figure 3.8).

The percentage of remaining fully innervated endplates was determined in every previously published and supplemented muscle from the *Smn*<sup>-/-</sup>;SMN2, Taiwanese, SMN $\Delta$ 7 and *Smn*<sup>2B/-</sup> mouse models. Each muscle was then classified with the seven-point colour-coded vulnerability classification tool for further analysis. For each of the four mouse models, all muscles were ordered based on decreasing numbers of fully innervated endplates (Figure 3.8). I found that in all four mouse models, the muscles appear to sit on a spectrum, displaying many different levels of vulnerability. There does not seem to be a bimodal split in relative vulnerability. Furthermore, there appears to be a similar range of levels in relative vulnerability across all four mouse models of SMA. In the *Smn*<sup>-/-</sup>;SMN2 mouse model, vulnerability levels range from 99.0% in the

adductor auris longus (Murray et al., 2008) to only 36.0% remaining fully innervated endplates in the caudal band of the levator auris longus (Thomson et al., 2012) (Figure 3.8A). Similarly, in the Taiwanese mouse model, the variability in vulnerability levels, ranges from 98.4% innervation in the semispinalis capitis to only 22.7% remaining innervation in the flexor digitorum brevis 2 (Lin et al., 2016) (Figure 3.8B). However, it is evident that the majority of muscles are comparatively resistant to synaptic loss in this model (100-91% fully innervated endplates). The Taiwanese mouse model displays the least overall vulnerability, with only six out of 26 investigated muscles showing a mild synaptic loss (90-71% fully innervated endplates), and only foot muscle FDB2 and FDB3 showing signs of a severe synaptic loss (<40% fully innervated endplates) in this model. In this comparison of consistency of vulnerability patterns, this makes the Taiwanese mice stand out as the model with the lowest levels of NMJ loss. The percentage of remaining innervated endplates in the SMN $\Delta$ 7 mouse models ranges from 100% in the quadriceps (Ling et al., 2012) to only 14.7% in the auricularis superior (Figure 3.8C). Lastly, in the *Smn*<sup>2B/-</sup> mouse model, innervation levels ranged from 100% innervated endplates in the adductor auris longus to only 29.3% remaining fully innervated endplates in the sternocleidomastoid (Figure 3.8D).

These findings taken together, show that all four investigated mouse models display a similar range of synaptic loss, approximately ranging from 30% to 100% remaining innervated endplates in each model. However, despite similar levels of NMJ loss throughout all four models, the Taiwanese mouse model shows a milder degree of NMJ pathology compared to the other three mouse models of SMA.

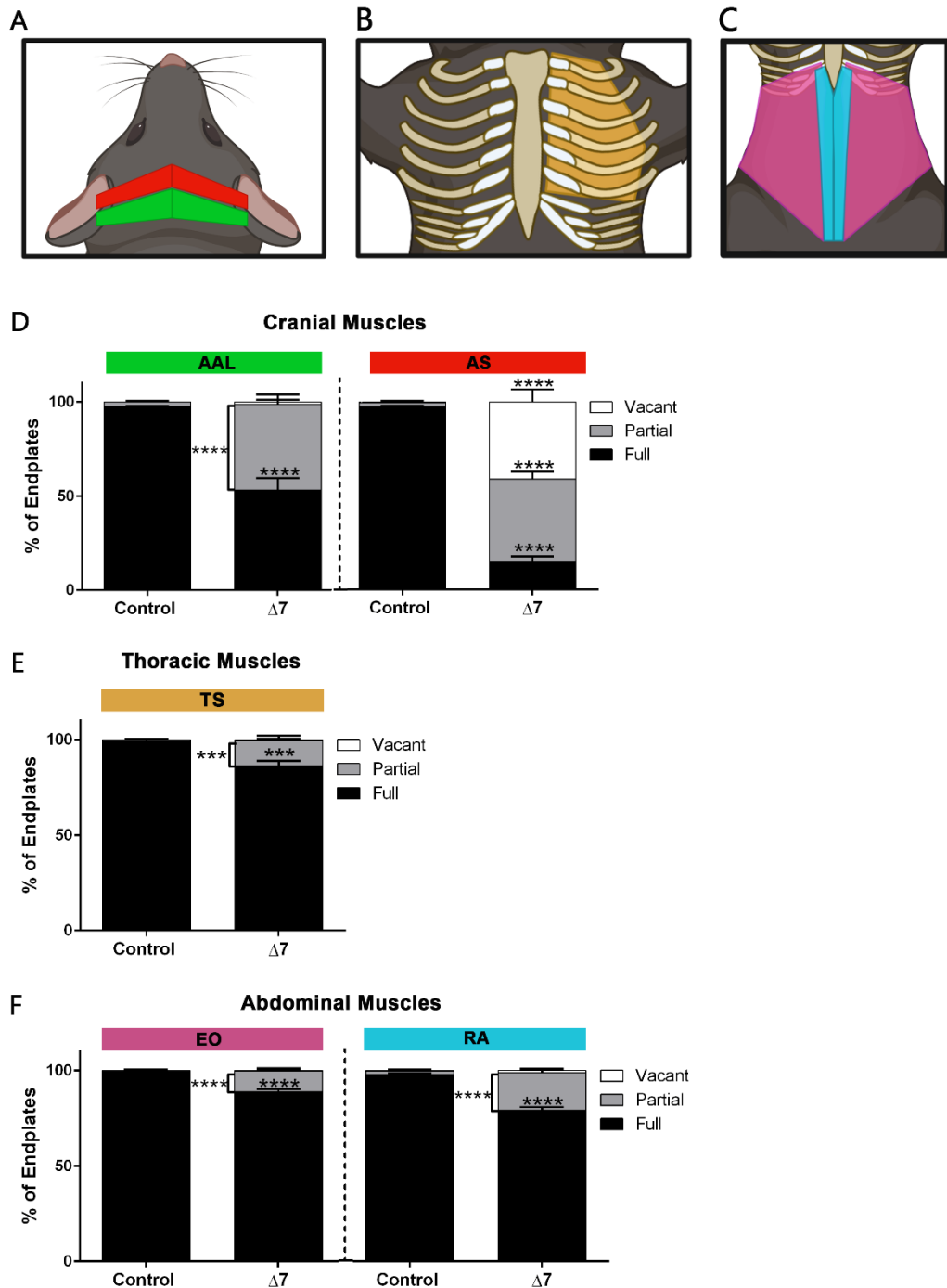


**Figure 3.7: Analysis of synaptic loss in cranial and thoracoabdominal muscles in SMN $\Delta 7$  mouse model at P12.** (A) Schematic diagram of the anatomical locations of cranial muscles (adductor auris longus [AAL] and auricularis superior [AS]) in a dorsally viewed mouse. (B) Schematic diagram of the anatomical locations of a thoracic muscle (triangularis sterni [TS]) in a mouse in supine position. (C) Schematic diagram of the anatomical locations of abdominal muscles (external oblique [EO] and rectus abdominis [RA]) in a mouse in supine position. (D-F) Bar charts showing the quantification of percentage of full, partial, and vacant endplates in SMN $\Delta 7$  mice compared to controls in cranial muscles, thoracic muscles, and abdominal muscles respectively. Note that all cranial muscles show a significant increase in vacant motor endplates and a significant decrease in fully occupied endplates, whereas none of the thoracic or abdominal muscles show a significant increase in vacant endplates. Two-sided ANOVA with Sidak correction (\*\* $p \leq 0.001$  and \*\*\*\* $p \leq 0.0001$ ).  $n=3$  for controls and SMN $\Delta 7$  mice respectively. 3 FOV quantified directly under the microscope for one muscle each per  $n$ -number. Error bars represent mean  $\pm$  SEM.

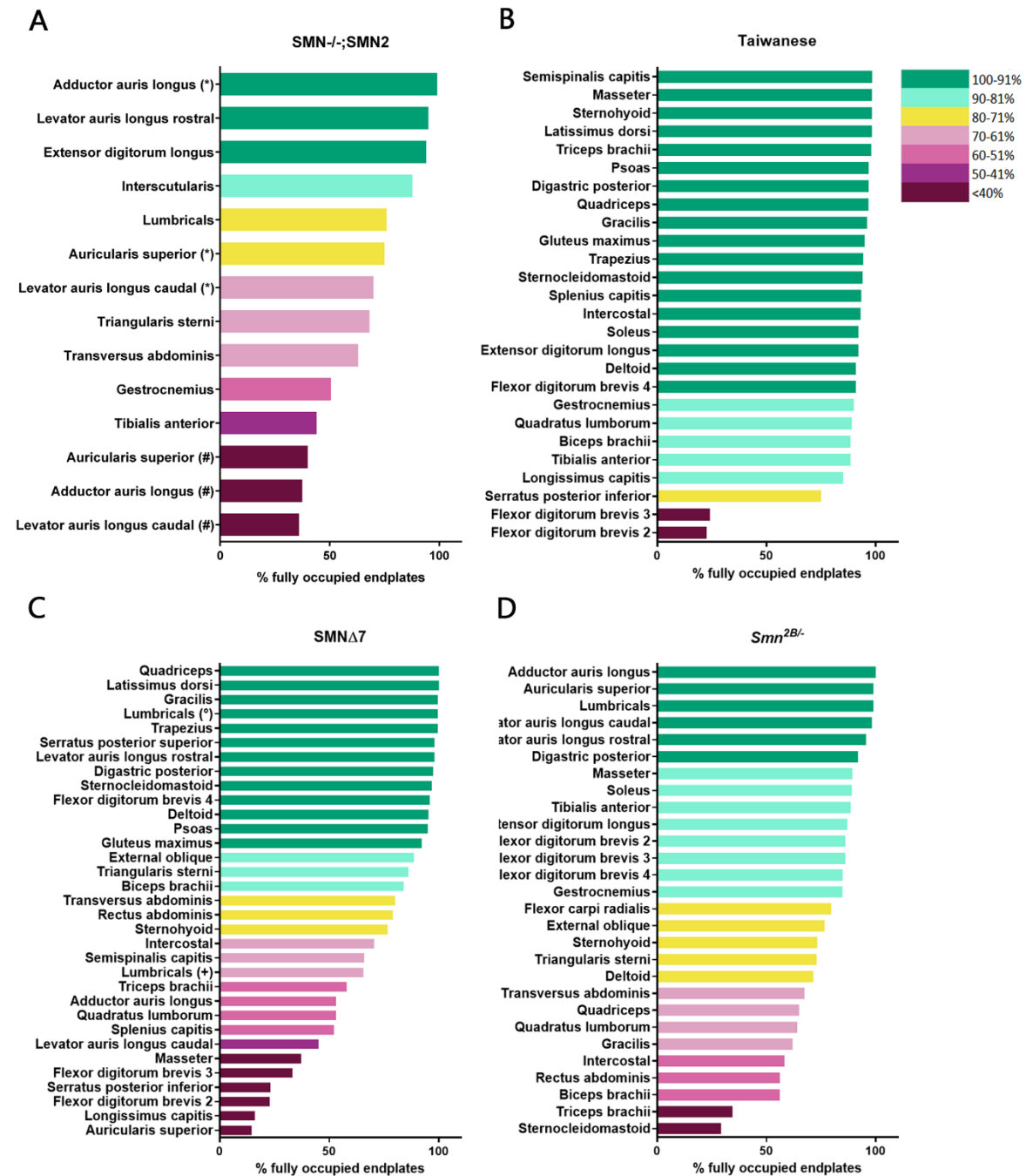


**Figure 3.8: Comparison of NMJ loss in four mouse models indicates a similar range of vulnerability.** (A–D) Bar charts showing the percentage of fully occupied endplates in previously investigated muscles in the *Smn*<sup>-/-</sup>;SMN2 (Murray et al., 2010a, 2008; Thomson et al., 2012); \* = Murray et al., 2010a, 2008; # = Thomson et al., 2012; Taiwanese (Buettner et al., 2021; Lin et al., 2016), SMNΔ7 (Buettner et al., 2021; Comley et al., 2016; Ling et al., 2012; Murray et al., 2008); ° = Ling et al., 2012; + = Comley et al., 2016, and *Smn*<sup>2B/-</sup> (Buettner et al., 2021; Murray et al., 2015) mouse model, respectively. The muscles in each mouse model are ordered by decreasing percentage of fully occupied endplates. Each muscle is coloured according to its classification category by percentage of fully occupied motor endplates present at end-stage of disease. Each of the four mouse models displays a wide variability in the percentage of NMJ loss and therefore in vulnerability levels. Note that the percentage of fully occupied endplates ranges from almost 100% to lowest values of only around 15–30% in all four models.

### 3.3.5. Comparison of synaptic loss reveals distinct patterns of selective vulnerability in the *Smn*<sup>-/-</sup>;SMN2, Taiwanese, SMNΔ7 and *Smn*<sup>2B/-</sup> mouse models

Next, I investigated the vulnerability pattern present in all four mouse models of SMA, by conducting comparisons between specific muscles across different mouse models. To gain a better understanding of the similarities and differences in the vulnerability patterns of these four mouse models, I used my own analysis and available published data to compare specific muscles (Figure 3.9). The table displays all previously investigated muscles in the *Smn*<sup>-/-</sup>;SMN2, Taiwanese, SMNΔ7 and *Smn*<sup>2B/-</sup> mouse models, categorised by anatomical body regions. The table shows a total of ten body regions, comprised of head, neck, back, thoracic, abdominal, proximal forelimb, distal forelimb, proximal hindlimb, distal hindlimb and foot muscles. Each muscle in the table is displayed in the colour of the allocated category of the seven-point colour-coded vulnerability classification tool, based on its number of remaining fully innervated endplates (see legend in Figure 3.9).

Analysis of this selective vulnerability data by body region revealed that the vulnerability of a given muscle varied considerably between mouse models (Figure 3.9). Firstly, it became evident that the regional pattern of vulnerability which is observed in the *Smn*<sup>2B/-</sup> mouse model and which showed increased synaptic loss in core and proximal limb muscles, was not seen in any of the other three models. In the Taiwanese mouse model, the majority of muscles displayed between 100% to 81% fully innervated endplates. The only exceptions with severe synaptic loss of <40% fully innervated endplates are two heads of the foot muscle flexor digitorum brevis (FDB 2 and FDB3). In contrast to the Taiwanese and *Smn*<sup>2B/-</sup> mouse model, many body regions in the *Smn*<sup>-/-</sup>;SMN2 and SMNΔ7 mouse models comprise of both vulnerable and resistant muscles within the same body region. In the SMNΔ7 mouse model, the frequency of this dichotomy of vulnerability in certain body regions seems to be particularly prominent. This dichotomy of vulnerability is especially pronounced in the head and neck muscles. In the head muscles, four muscles were extremely vulnerable (AAL, AS, LALc and MA), whilst two were largely unaffected (LALr and DP) by pathology. This is especially interesting, since all

of these head muscles are innervated by the same nerve, the cranial nerve VII. A similar picture was observed in the neck muscles of the SMN $\Delta$ 7 mouse model. Here, three muscles showed extremely high levels of NMJ loss (longissimus capitis, serratus posterior inferior and splenius capitis) and two were moderately affected (semispinalis capitis and SH), while two muscles were largely unaffected (serratus posterior superior and SCM) by NMJ pathology.

These profound differences in vulnerability patterns between mouse models of SMA can best be highlighted by looking at some examples of how individual muscles differ across models. For example, the three cranial muscles AS, AAL and LALc are severely affected with high levels of synaptic loss in the SMN $\Delta$ 7 model and the *Smn*<sup>-/-</sup>;SMN2 mouse models, but they are resistant in the *Smn*<sup>2B/-</sup> mouse model with almost all their endplates still innervated. A further example of the differences in innervation between mouse models is the triceps brachii (TC) muscle. In the Taiwanese model, almost all innervation is remaining in the TC, while it is moderately affected in the SMN $\Delta$ 7 model with 57.9% endplates remaining fully innervated, and a severe synaptic loss with only 34.4% remaining innervated endplates in the *Smn*<sup>2B/-</sup> mouse model. The TA muscle, a distal hindlimb muscle, is 88.4% and 88.5% fully occupied in the Taiwanese and *Smn*<sup>2B/-</sup> mouse models respectively, but only 44.0% fully innervated endplates remained in the *Smn*<sup>-/-</sup>;SMN2 mouse model. Further, the thoracic intercostal muscles (IC) are also differentially affected across all models. While they are moderately affected in the *Smn*<sup>2B/-</sup> and SMN $\Delta$ 7 models with 58.1% and 70.4% of fully innervated endplates remaining respectively, there is almost no synaptic loss present in the Taiwanese model with 93.0% remaining fully innervated endplates.

| Vulnerable and Resistant Muscles in Mouse Models of SMA |                          |           |       |                     |  |
|---------------------------------------------------------|--------------------------|-----------|-------|---------------------|--|
| Muscle Name                                             | SMN <sup>-/-</sup> ;SMN2 | Taiwanese | SMNΔ7 | SMN <sup>2B/-</sup> |  |
| Head Muscles                                            |                          |           |       |                     |  |
| Adductor auris longus (AAL)                             | *                        | #         |       |                     |  |
| Auricularis superior (AS)                               | *                        | #         |       |                     |  |
| Interscutularis (IS)                                    |                          |           |       |                     |  |
| Levator auris longus caudal (LALc)                      | *                        | #         |       |                     |  |
| Levator auris longus rostral (LALr)                     |                          |           |       |                     |  |
| Digastric posterior                                     |                          |           |       |                     |  |
| Masseter                                                |                          |           |       |                     |  |
| Neck Muscles                                            |                          |           |       |                     |  |
| Longissimus capitis                                     |                          |           |       |                     |  |
| Semispinalis capitis                                    |                          |           |       |                     |  |
| Serratus posterior inferior (SPI)                       |                          |           |       |                     |  |
| Serratus posterior superior (SPS)                       |                          |           |       |                     |  |
| Splenius capitis                                        |                          |           |       |                     |  |
| Sternocleidomastoid                                     |                          |           |       |                     |  |
| Sternohyoid                                             |                          |           |       |                     |  |
| Back Muscles                                            |                          |           |       |                     |  |
| Latissimus dorsi                                        |                          |           |       |                     |  |
| Quadratus lumborum                                      |                          |           |       |                     |  |
| Trapezius                                               |                          |           |       |                     |  |
| Thoracic Muscles                                        |                          |           |       |                     |  |
| Intercostal (IC)                                        |                          |           |       |                     |  |
| Triangularis sterni (TS)                                |                          |           |       |                     |  |
| Abdominal Muscles                                       |                          |           |       |                     |  |
| External oblique (EO)                                   |                          |           |       |                     |  |
| Rectus abdominis (RA)                                   |                          |           |       |                     |  |
| Transversus abdominis (TVA)                             |                          |           |       |                     |  |
| Proximal Forelimb Muscles                               |                          |           |       |                     |  |
| Biceps brachii (BC)                                     |                          |           |       |                     |  |
| Deltoid                                                 |                          |           |       |                     |  |
| Triceps brachii (TC)                                    |                          |           |       |                     |  |
| Distal Forelimb Muscles                                 |                          |           |       |                     |  |
| Flexor carpi radialis (FCR)                             |                          |           |       |                     |  |
| Proximal Hindlimb Muscles                               |                          |           |       |                     |  |
| Gluteus maximus                                         |                          |           |       |                     |  |
| Gracilis                                                |                          |           |       |                     |  |
| Quadriceps (QC)                                         |                          |           |       |                     |  |
| Psoas                                                   |                          |           |       |                     |  |
| Distal Hindlimb Muscles                                 |                          |           |       |                     |  |
| Extensor digitorum longus (EDL)                         |                          |           |       |                     |  |
| Gastrocnemius (GS)                                      |                          |           |       |                     |  |
| Soleus (SO)                                             |                          |           |       |                     |  |
| Tibialis anterior (TA)                                  |                          |           |       |                     |  |
| Foot Muscles                                            |                          |           |       |                     |  |
| Flexor digitorum brevis-2 (FDB2)                        |                          |           |       |                     |  |
| Flexor digitorum brevis-3 (FDB3)                        |                          |           |       |                     |  |
| Flexor digitorum brevis-4 (FDB4)                        |                          |           |       |                     |  |
| Lumbricals                                              |                          |           |       |                     |  |

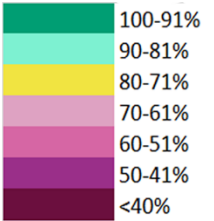


**Figure 3.9: Comparison of NMJ loss in four mouse models indicates diverse patterns of selective vulnerability.** The table shows investigated muscles coloured according to their classification categories by percentage of fully occupied endplates at disease end-stage in the *Smn<sup>-/-</sup>;SMN2* (Murray et al., 2010a, 2008; Thomson et al., 2012), Taiwanese (Buettner et al., 2021; Lin et al., 2016), *SMNΔ7* (Buettner et al., 2021; Comley et al., 2016; Ling et al., 2012; Murray et al., 2008) and *Smn<sup>2B/-</sup>* (Buettner et al., 2021; Murray et al., 2015) mouse model. The table displays the muscles categorised by body regions. Note that for some muscles, different

results were observed in two independent studies. This occurred in the AAL, AS and LALc muscles \* = Murray et al., 2010a, 2008; # = Thomson et al., 2012 in the *Smn*<sup>-/-</sup>;SMN2 model and the LU + = Comley et al., 2016; ° = Ling et al., 2012 in the SMNΔ7 model. In the *Smn*<sup>-/-</sup>;SMN2 mouse model, all body regions seem to be moderately affected and display different vulnerability levels, with the most severely affected the muscles in the hindlimb. The Taiwanese mouse model shows very little vulnerability, except for the FDB 2 and FDB 3. The vulnerability pattern in the SMNΔ7 mouse model shows that the head, neck and foot muscles are the most affected, followed by ventral core muscles and the least vulnerability is seen in the limb and back muscles. The *Smn*<sup>2B/-</sup> mouse model displays a regional vulnerability pattern, where the neck muscles, core muscles and proximal limbs seem more affected than the head muscles, distal limbs and foot muscles.

The data demonstrated that the displayed selective vulnerability patterns are extremely variable between the most commonly utilised mouse models of SMA, when categorised by anatomical body region. In order to explore other factors which may affect relative vulnerability, the muscles were re-grouped (Figure 3.10). Pun et al. found that skeletal muscles can be subdivided into two distinct categories of muscles, called delayed synapsing (DeSyn) and fast synapsing (FaSyn) (Pun et al., 2002). These two muscle categories possess intrinsically distinct features of focal endplate (AChR) clustering. They also differ in the rate at which they achieve the specific and characteristic organisational structure of a matured NMJ. While FaSyn muscles achieve focal organisation within a single day, DeSyn muscles take up to five days to reach the organisation of mature NMJs (Pun et al., 2002). It was previously hypothesised that FaSyn muscles may be more prone to vulnerability in SMA-induced loss of NMJs (Murray et al., 2008). However, it was also previously demonstrated that several FaSyn muscles from the SMNΔ7 mouse model were relatively resistant to SMA-induced synaptic loss (Ling et al., 2012). To investigate if the rate of synapsing could be a factor which affects relative vulnerability, muscles from the four most commonly utilised mouse models of SMA were grouped based upon their fast synapsing (FaSyn) and delayed synapsing (DeSyn) phenotype (Figure 3.10A). The data revealed that DeSyn muscles in the Taiwanese and SMNΔ7 mouse model were relatively resistant, while DeSyn muscles in the *Smn*<sup>-/-</sup>;SMN2 and *Smn*<sup>2B/-</sup> mouse model included both resistant and vulnerable muscles. In comparison, the FaSyn muscles showed a mixed vulnerability in all mouse models with the exception of the Taiwanese model, where FaSyn muscles were solely resistant. Overall, the data

suggested no correlation between relative vulnerability and synapsing phenotype (Figure 3.10A).

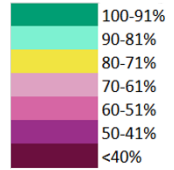
Next, muscles were grouped according to their muscle fibre type to investigate if this factor could affect the relative vulnerability in mouse models of SMA (Figure 3.10B). Muscles fibre types can be subdivided into slow twitch, fast twitch and mixed fibre types, according to the physiological properties of the motor neurons that innervate them. It was previously proposed that muscle fibre types render vulnerability of the synapses in amyotrophic lateral sclerosis, where muscles comprised of fast-twitch muscle fibres were more vulnerable to degeneration during disease (Pun et al., 2006). After grouping muscles based on muscle fibre type, data showed that there was no obvious correlation between muscle fibre type and relative vulnerability of muscles in any of the four investigated mouse models of SMA (Figure 3.10B). In all three muscle fibre categories (fast-twitch, mixed fibres and slow-twitch), some muscles showed high vulnerability, some muscles were mildly or moderately affected, and some muscles were resistant throughout all mouse models. These findings are in line with previous investigation on the correlation between muscle fibre type and relative vulnerability in mouse models of SMA. Previous work in the *SMN $\Delta$ 7* mouse model revealed no simple correlation between relative vulnerability and muscle fibre type (Ling et al., 2012). A similar conclusion was drawn from investigations in the *Smn<sup>-/-</sup>;SMN2* mouse model of SMA, where both fast twitch and slow twitch muscles were shown to be vulnerable to degeneration (Murray et al., 2010a, 2008). Taken together, the findings revealed that neither synapsing type nor muscle fibre type showed a correlation to relative vulnerability of muscles in any of the four most commonly utilised mouse models of SMA.

The findings described in this section confirm that the development of the new seven-point colour-coded vulnerability classification tool facilitated the detailed analysis of vulnerability patterns. The results show that the comparison of selective vulnerability patterns revealed four distinct patterns of selective vulnerability in commonly used mouse models of SMA. The discovery of profound differences in vulnerability patterns between mouse models of SMA

is very important to consider during experimental design. Choosing a muscle or body region in a mouse model of interest that meets specific requirements for the experimental design is imperative for meaningful interpretation of the generated findings. Selective vulnerability levels can be utilised as a tool to better understand the mechanisms driving selective vulnerability in different mouse models of SMA. However, further work on this topic is needed to uncover the mechanisms underlying NMJ and motor neuron vulnerability in these mouse models of SMA.

**A**

| Vulnerable and Resistant Muscles in Mouse Models of SMA |                          |           |       |  |
|---------------------------------------------------------|--------------------------|-----------|-------|--|
| Muscle Name                                             | SMN <sup>-/-</sup> ;SMN2 | Taiwanese | SMNΔ7 |  |
| DeSyn                                                   |                          |           |       |  |
| Levator auris longus rostral (LALr)                     |                          |           |       |  |
| Sternocleidomastoid                                     |                          |           |       |  |
| Gluteus maximus                                         |                          |           |       |  |
| Gracilis                                                |                          |           |       |  |
| Gastrocnemius (GS)                                      |                          |           |       |  |
| Soleus (SO)                                             |                          |           |       |  |
| FaSyn                                                   |                          |           |       |  |
| Levator auris longus caudal (LALc)                      | *                        |           |       |  |
| Intercostal (IC)                                        |                          |           |       |  |
| Quadriceps (QC)                                         |                          |           |       |  |
| Extensor digitorum longus (EDL)                         |                          |           |       |  |
| Tibialis anterior (TA)                                  |                          |           |       |  |



**B**

| Vulnerable and Resistant Muscles in Mouse Models of SMA |                          |           |       |                     |
|---------------------------------------------------------|--------------------------|-----------|-------|---------------------|
| Muscle Name                                             | SMN <sup>-/-</sup> ;SMN2 | Taiwanese | SMNΔ7 | Smn <sup>2B/-</sup> |
| Fast twitch                                             |                          |           |       |                     |
| Adductor auris longus (AAL)                             | *                        | #         |       |                     |
| Levator auris longus caudal (LALc)                      | *                        | #         |       |                     |
| Levator auris longus rostral (LALr)                     |                          |           |       |                     |
| Digastric posterior                                     |                          |           |       |                     |
| Masseter                                                |                          |           |       |                     |
| Longissimus capitis                                     |                          |           |       |                     |
| Serratus posterior inferior (SPI)                       |                          |           |       |                     |
| Serratus posterior superior (SPS)                       |                          |           |       |                     |
| Splenius capitis                                        |                          |           |       |                     |
| Sternocleidomastoid                                     |                          |           |       |                     |
| Sternohyoid                                             |                          |           |       |                     |
| Latissimus dorsi                                        |                          |           |       |                     |
| Intercostal (IC)                                        |                          |           |       |                     |
| Biceps brachii (BC)                                     |                          |           |       |                     |
| Deltoid                                                 |                          |           |       |                     |
| Triceps brachii (TC)                                    |                          |           |       |                     |
| Gluteus maximus                                         |                          |           |       |                     |
| Psoas                                                   |                          |           |       |                     |
| Extensor digitorum longus (EDL)                         |                          |           |       |                     |
| Tibialis anterior (TA)                                  |                          |           |       |                     |
| Flexor digitorum brevis-2 (FDB2)                        |                          |           |       |                     |
| Flexor digitorum brevis-3 (FDB3)                        |                          |           |       |                     |
| Flexor digitorum brevis-4 (FDB4)                        |                          |           |       |                     |
| Lumbricals                                              |                          |           | +     | o                   |
| Mixed                                                   |                          |           |       |                     |
| Semispinalis capitis                                    |                          |           |       |                     |
| Trapezius                                               |                          |           |       |                     |
| Gracilis                                                |                          |           |       |                     |
| Quadriceps (QC)                                         |                          |           |       |                     |
| Gastrocnemius (GS)                                      |                          |           |       |                     |
| Slow twitch                                             |                          |           |       |                     |
| Auricularis superior (AS)                               | *                        | #         |       |                     |
| Triangularis sterni (TS)                                |                          |           |       |                     |
| Transversus abdominis (TVA)                             |                          |           |       |                     |
| Soleus (SO)                                             |                          |           |       |                     |

**Figure 3.10: Comparison of intrinsic muscle properties in four mouse models of SMA.** The table shows investigated muscles coloured according to their classification categories by percentage of fully occupied endplates at disease end-stage in the *Smn<sup>-/-</sup>;SMN2* (Murray et al., 2010a, 2008; Thomson et al., 2012), Taiwanese (Buettner et al., 2021; Lin et al., 2016), SMNΔ7 (Buettner et al., 2021; Comley et al., 2016; Ling et al., 2012; Murray et al., 2008) and *Smn<sup>2B/-</sup>* (Buettner et al., 2021; Murray et al., 2015). **(A)** The table displays the muscles categorised by delayed synapsing (DeSyn) or fast synapsing (FaSyn). Note that for the LALc

muscle, different results were observed in two independent studies (\* = Murray et al., 2008; # = Thomson et al., 2012). **(B)** The table displays the muscles categorised by muscle fibre type (fast-twitch, mixed fibre types and slow-twitch). Note that for the AS, AAL, LALc and lumbrical muscles, different results were observed in two independent studies (\* = Murray et al., 2010a, 2008; # = Thomson et al., 2012; + = Comley et al., 2016; ° = Ling et al., 2012).

### **3.3.6. *Skeletal Muscle* Publication Declaration**

Some of the work presented within this chapter of this thesis has been published as a research paper in *Skeletal Muscle*. The publication is entitled: Mouse models of SMA show divergent patterns of neuronal vulnerability and resilience (Woschitz et al., 2022). A copy of this publication is provided as Appendix 1 of this thesis.

### 3.4. Discussion

#### 3.4.1. Overview of results

In this chapter of this thesis, I investigated selective vulnerability patterns of the four most commonly utilised mouse models of SMA to get a deeper insight into the consistency of vulnerability patterns between these models. I firstly conducted a thorough body wide analysis and characterisation of NMJ and motor neuron cell body loss in the *Smn*<sup>2B/-</sup> mouse model at disease end-stage. I showed that there is a distinct regional vulnerability pattern present in this model, with increased vulnerability in the centre of the spinal cord. In this model, muscles that are more prone to vulnerability include core, proximal forelimb and proximal hindlimb muscles. I further showed that the increased vulnerability in the cervical, thoracic, and upper lumbar levels of the spinal cord correlates with an increased loss of motor neuron cell bodies in the thoracic spinal cord regions. Comparison of selective vulnerability patterns in four most commonly utilised mouse models of SMA showed that each mouse model presented with a similar range of NMJ loss (30-100%). Despite this, each model displayed a distinct pattern of selective vulnerability. The Taiwanese model showed the least NMJ pathology, while the *Smn*<sup>-/-</sup>; *SMN2* and *SMNΔ7* showed a high frequency of dichotomy of vulnerability throughout body regions. The results of this chapter demonstrate that each mouse model of SMA has a distinct pattern of vulnerability, while displaying the same range of NMJ loss in all models.

#### 3.4.2. Contributing factors of differential vulnerability in the four most commonly used mouse models of SMA

I conducted a thorough body wide analysis on the selective vulnerability pattern of muscles in the *Smn*<sup>2B/-</sup> mouse model of SMA and demonstrated that this model displays a unique regional vulnerability pattern. Each body region seems to consist solely of either comparatively resistant or solely of comparatively vulnerable muscles. This regional vulnerability pattern observed in the *Smn*<sup>2B/-</sup> mouse model is a feature that was previously documented in SMA patients. Patients commonly present with an increased vulnerability and an earlier weakness and atrophy in muscles of the core, proximal leg and proximal arm compared to muscles of the distal arm and distal leg (Deymeer

et al., 2008; Dubowitz, 1999; Wadman et al., 2018). The vulnerability pattern of the *Smn*<sup>2B/-</sup> mouse model presents with a sparing of muscles innervated by the CN VII and by lower lumbar spinal nerves, thereby rendering head and distal hindlimb muscles as relatively resistant to degeneration. Further, an increased vulnerability was observed in muscles of the neck, core, proximal forelimb and the proximal hindlimb. This pattern has previously also been observed in patients with SMA (Dubowitz, 1999; Wadman et al., 2018), suggesting that the disease progresses segmentally starting with the upper lumbar segments (Deymeer et al., 2008). With increasing in-depth knowledge about vulnerability patterns in mouse models of SMA, it will become increasingly important to conduct a comprehensive study that profiles the selective vulnerability pattern present in patients. This would allow the comparison of differences and similarities between human disease and animal models of disease. However, the lack of such a study is not surprising, as it is challenging to investigate vulnerability patterns in autopsy materials. Firstly, the temporal order of motor neuron loss cannot be detected from autopsy material, and secondly, human autopsy materials usually reflect a later disease stage than mice do at 'end-stage', which is due to clinical intervention and care. Regardless, the remarkable similarities between the observed vulnerability patterns in SMA patients and the regional vulnerability pattern in the *Smn*<sup>2B/-</sup> mouse model is intriguing, making this a valuable model for further research studies. These findings support the use of this mouse model in the identification of potential neuroprotective modifiers of disease which could have strong clinical relevance.

The body wide analysis of NMJ loss in the *Smn*<sup>2B/-</sup> mouse model that was presented here, showed that muscles innervated by the centre of the spinal cord were more vulnerable than muscles innervated by cranial nerves and lower lumbar spinal nerves. However, the reasons for the regional pattern of selective vulnerability are unclear. In ALS it has previously been shown that muscle fibre type seems to be a key regulator of selective vulnerability (Nijssen et al., 2017; Pun et al., 2006). Muscles fibre types can be subdivided into slow twitch, fast twitch and mixed fibre types, according to the physiological properties of the motor neurons that innervate them. Pun et al. showed that

muscles comprised of fast-twitch muscle fibres and innervated by fast-fatigable motor neurons were vulnerable in ALS, while slow-twitch muscles innervated by slow motor neurons were resistant to degeneration (Pun et al., 2006). Here, data showed no obvious correlation between muscle fibre type and relative vulnerability of muscles in any of the four investigated mouse models of SMA. These findings are consistent with previous investigations in the  $SMN\Delta 7$  mouse model, revealing no simple correlation between relative vulnerability and muscle fibre type (Ling et al., 2012). Similar conclusions were drawn from investigations in the  $Smn^{-/-};SMN2$  mouse model of SMA, which showed that both, fast twitch and slow twitch muscles, were vulnerable to degeneration (Murray et al., 2010a, 2008).

Previous work further suggested that the development and stability of NMJs is a contributing factor to selective vulnerability (Murray et al., 2008; Pun et al., 2002). Skeletal muscles can be subdivided into two distinct categories of muscles, called delayed synapsing (DeSyn) and fast synapsing (FaSyn), based upon their synapsing phenotype (Pun et al., 2002). FaSyn muscles were found to be more vulnerable in head muscles of a SMA mouse model than DeSyn muscles (Murray et al., 2008). It was also previously demonstrated that several FaSyn muscles from the  $SMN\Delta 7$  mouse model were relatively resistant to SMA-induced synaptic loss (Ling et al., 2012). Here, data suggested no correlation between relative vulnerability and synapsing phenotype, which is in line with the contrasting findings from previous studies in different mouse models of SMA (Ling et al., 2012; Murray et al., 2008).

Lastly, it was previously proposed that the location of motor neurons within the spinal cord could be a regulating factor of vulnerability in mouse models of SMA (Mentis et al., 2011; d'Errico et al., 2013). Motor neuron loss was found to be most pronounced in medial motor neurons innervating axial and proximal limb muscles and less pronounced in medial motor neurons innervating distal limb muscles (d'Errico et al., 2013; Mentis et al., 2011). Mentis et al. showed that in the  $SMN\Delta 7$  mouse model there was an increased breakdown of sensory motor neurons at spinal cord level L1 which innervates the proximal hindlimb, than at L5, which innervates the distal hindlimb (Mentis et al., 2011). Further,

motor neurons innervating the proximal forelimb were shown to display selective shrinkage in comparison to motor neurons innervating the distal forelimb (d'Errico et al., 2013). Data in this chapter shows that there is a similar pattern of selective vulnerability present in the *Smn*<sup>2B/-</sup> mouse model. Here, axial, proximal forelimb, and proximal hindlimb muscles showed the highest levels of vulnerability. Findings from the four-model comparison indicated that this pattern of vulnerability is not a feature present across all SMA mouse models. However, the correlation between anatomical muscle location and vulnerability in the *Smn*<sup>2B/-</sup> mouse model and in SMA patients seems intriguing. Further research into the intrinsic molecular processes of differentially vulnerable muscles will be required to reliably identify contributing factors of selective vulnerability.

### **3.4.3. Dramatic difference in vulnerability patterns between mouse models of SMA**

In this chapter I compared the synaptic loss of muscles throughout ten different body regions to assess similarities and differences in patterns of selective vulnerability in the four most commonly used mouse models of SMA. I showed that each model displays a distinct pattern of selective vulnerability. These findings are consistent with published work on muscles compared between three different mouse models of SMA (Buettner et al., 2021). The distal limb muscle TA was less vulnerable across all three models, while the back muscle quadratus lumborum varied between 45% to 100% remaining innervation in the three compared mouse models of SMA (Buettner et al., 2021). To mimic the phenotype of human spinal muscular atrophy, all of the investigated mouse models share a mutation within the *Smn* gene (Bowerman et al., 2012a; Hsieh-Li et al., 2000; Le et al., 2005; Monani et al., 2000). Despite this, there is a drastic difference between the observed vulnerability patterns of muscles between models. The reasons for these vast differences remain unclear, but comparing the selective vulnerability patterns of different mouse models of SMA has the potential to make a valuable contribution to further understanding the mechanisms that drive motor neuron pathology in SMA. One explanation for the observed differences could be rooted in the variations in underlying genetics of the four investigated mouse models. Copies of the human *SMN2*

transgene have been inserted in a non-native position to produce low levels of Smn protein in the *Smn*<sup>-/-</sup>;SMN2, Taiwanese and SMNΔ7 mouse models, thereby potentially causing positional effects in the expression of Smn levels. In contrast, the *Smn*<sup>2B/-</sup> mouse model is the only model which produces reduced Smn levels that come from a gene in its native position. Another possible explanation for the differences in motor neuron vulnerability could be the varying relative Smn levels between models. Western blot analysis revealed that Smn protein levels in spinal cord are lower in SMNΔ7 mice compared to Taiwanese mice and the *Smn*<sup>2B/-</sup> mouse model (Buettner et al., 2021). However, while relative Smn levels are similar between the Taiwanese and the *Smn*<sup>2B/-</sup> mouse model (Buettner et al., 2021), their displayed patterns of selective vulnerability are diverse. Previous work has identified motor neuron loss in the Taiwanese model as comparatively milder (Buettner et al., 2021; Thomson et al., 2012), which is consistent with the findings of relatively resistant muscles throughout this model, which was presented in this chapter. Relative Smn levels can therefore not account for the differences between vulnerability patterns. While there has been some investigation of the relative Smn levels between mouse models of SMA, a comprehensive analysis of the expression of the *Smn* gene and the Smn protein products along the neuraxis is lacking, with only a few publications that started to investigate this matter. One study found that there are higher Smn protein levels in brain and spinal cord, in both the Taiwanese and the *Smn*<sup>2B/-</sup> mouse model of SMA, compared to other non-neuronal tissue types (Groen et al., 2018). However, the protein levels were measured in whole tissues rather than in separate spinal cord segments or levels. A recent autopsy study on SMA patient samples and control samples, revealed higher SMN protein levels in the thoracic and lumbar spinal cord regions during foetal development, than during postnatal development (Ramos et al., 2019). They also found that SMN protein expression correlates with full-length *SMN1* mRNA prenatally, but not postnatally in human spinal cord. (Ramos et al., 2019). Further work will be required to better understand why motor neuron pathology differs between mouse models of SMA. The findings in this chapter support the differences in vulnerability pattern of different mouse models, highlighting the importance to

consider the impact of the chosen model and experimental design on conclusions.

Given that all mouse models vary in their age of onset, one possible contributor to the difference in selective vulnerability pattern in the *Smn*<sup>2B/-</sup> mouse model, could be postnatal age. Previous work has shown that different muscles mature at different rates and that the postnatal age of mice can influence pathways that are responsible for the regeneration of axons (Kline et al., 2019a; Mole et al., 2020; Murray et al., 2011). During the first postnatal month, the neuromuscular system undergoes dynamic molecular and structural changes associated with postnatal maturation. These changes include developmental synapse elimination and consolidation and maturation of remaining connections. Recent work has also shown that pathways regulating synaptic and axonal degeneration following traumatic or hypoxic injury are also developmentally regulated, with a dramatic acceleration in the rate of degeneration following injury (Mole et al., 2020; Murray et al., 2011). It has been shown that postnatal changes in mitochondria are responsible for this acceleration in axon degeneration following injury. Findings showed that mitochondria was progressively enriched in both muscle and peripheral nerve axons during the first postnatal month (Kim et al., 2019; Kline et al., 2019a). Interestingly, bioenergetics status has also previously been proposed as a regulating factor of selective vulnerability in mouse models of SMA (Boyd et al., 2017). One reason for the development of different selective vulnerability patterns could be the developmental regulation of neuronal tissue dynamics. This could play an important role since all investigated mouse models display a different age of onset, ranging from P0 to P10. A hypothesis as to why the patterns of selective vulnerability are so distinct across mouse models of SMA can be inferred from the above discussed considerations. The different postnatal age of onset in each model, combined with the multiple processes that regulate muscle and neuronal development, could provide an explanation for the vast differences in selective vulnerability patterns between mouse models. Further work will be required to uncover the reasons for these vast differences in selective vulnerability patterns in mouse models of SMA. However, investigating the way mitochondria change in synapses, axons,

motor neuron cell bodies and motor units, has the potential to reveal reasons for the observed differences in vulnerability patterns between SMA mouse models.

#### **3.4.4. Conclusions**

Overall, the work presented here represents a detailed analysis of selective vulnerability patterns in the four most commonly used mouse models of SMA. Each of the investigated mouse models demonstrates a distinct pattern of selective vulnerability, with the *Smn*<sup>2B/-</sup> mouse model displaying a regional vulnerability pattern that shows remarkable similarities with the vulnerability pattern from SMA patients. Taken together, the findings suggest that selective vulnerability can be used as a tool to determine an appropriate experimental design for specific research questions. More work is required to shed light on the reasons why there are such vast differences in selective vulnerability patterns between mouse models of SMA.

## Chapter 4

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### Identification of potential neuroprotective modifiers through transcriptional analysis in mouse models of SMA

#### 4.1. Chapter overview and key findings

In this chapter, I aimed to identify potential neuroprotective modifiers by conducting transcriptional analysis of differentially vulnerable motor neuron populations in two mouse models of SMA. Here, I compared published data sets from two different screens on differentially vulnerable motor neurons to produce refined lists of candidate neuroprotective modifiers. In a first comparison, I identified a transcriptional signature unique to the resistant motor neurons of the CN VII nucleus in the *Smn*<sup>2B/-</sup> mouse model. In a second comparison, I identified transcripts which show consistent changes in wildtype motor neurons in populations of cells which are known to be vulnerable in SMA. Since RNA-sequencing analysis indicated PACAP as potential modifier associated with motor neuron protection, I further investigated its neuroprotective potential in the *Smn*<sup>2B/-</sup> mouse model of SMA. I treated *Smn*<sup>2B/-</sup> mice and their control littermates with PACAP, using IP injections of purified PACAP27 or PACAP38 protein or a single ICV injection of AAV9-PACAP-GFP to increase PACAP levels and to determine the effect on phenotype and neuropathology. Overall, this chapter provides a cross-model comparison of differently vulnerable motor neuron populations in healthy and SMA animals at pre-symptomatic stages of their disease onset, identifying and testing potential neuroprotective modifiers of SMA.

The principal findings of the following chapter are:

- Analysis of RNA-seq data sets from CN VII motor neurons in SMN $\Delta$ 7 and the *Smn*<sup>2B/-</sup> mouse model reveals an enrichment for transcripts falling within the gene ontology terms ‘protein localisation to cell periphery’ and ‘ribonucleoprotein complex biogenesis’.

- This comparison reveals novel transcripts upregulated in selectively resistant motor neurons, including those involved with axonal transport, RNA processing and mitochondrial bioenergetics.
- Analysis of RNA-sequencing data sets from differentially vulnerable motor neuron populations in neurologically normal control animals of the SMN $\Delta$ 7 and the *Smn*<sup>2B/-</sup> mouse model yielded a refined list of potentially neuroprotective modifiers including *Col5a2*, *Gabra1*, *Gira1* and *Adcyap1*.
- IP injected PACAP protein has no significant effect on weight, motor phenotype and levels of synaptic loss in the *Smn*<sup>2B/-</sup> mouse model of SMA.
- ICV injected AAV9-PACAP-GFP has no significant effect on levels of synaptic loss in the *Smn*<sup>2B/-</sup> mouse model of SMA.

## 4.2. Introduction

In SMA, it has been well described that not all motor neurons show uniform levels of vulnerability to degeneration during disease progression. Some motor neurons remain intact until late stages of the disease, while others are lost early during the course of the disease. This phenomenon of selective vulnerability has long been recognised in SMA patients as well as in mouse models of the disease. However, the reasons and underlying mechanisms of this differential resilience of motor neurons in SMA are still unclear.

In Chapter 3, I compared patterns of selective vulnerability across SMA mouse models, showing that differential vulnerability can be utilised as tool to better tailor future experimental designs in SMA research. The phenomenon of selective vulnerability further provides an opportunity to investigate mechanisms that can drive motor neuron protection in SMA, which can in turn reveal potentially interesting modifiers of disease. This approach has previously been utilised by various research groups to identify modifiers of motor neuron pathology in different motor neuron diseases. In ALS, several studies utilised differentially vulnerable motor neuron populations from post-mortem tissues of neurologically normal mice, rats and humans to perform analysis of their global transcriptome. These transcriptional analysis led to the identification of IGF-II (Hedlund et al., 2010), GABA and glutamate receptors (Brockington et al., 2013), MMP-9 (Kaplan et al., 2014), and SYT13 (Nizzardo et al., 2020) as important disease modifiers in ALS.

Transcriptional profiling of differentially vulnerable motor neuron populations from different mouse models has previously also been utilised in SMA to identify modifiers of disease. Here, DNA repair transcripts (Murray et al., 2015), *Pgk1* (Boyd et al., 2017), and *Gdf-11* (Nichterwitz et al., 2020) were identified as neuroprotective modifiers. Murray et al. have utilised vulnerable spinal cord motor neurons and resistant CN VII (facial nucleus) motor neurons to perform a four-way comparison in neurologically normal (wildtype) and *Smn*<sup>2B/-</sup> mice at a pre-symptomatic time point P10 (Murray et al., 2015). This comparison of differentially vulnerable motor neuron populations between *Smn*<sup>2B/-</sup> and control animals demonstrated that a reduction in Smn levels correlates with a

decrease in transcripts that are associated with the ribosome, rRNA binding, and ubiquitin and oxidative phosphorylation. They further showed that vulnerable motor neurons demonstrate a decrease in markers that are associated with DNA repair (Murray et al., 2015). Previous work by Nichterwitz et al. utilised discrete neuronal population throughout the CNS from wildtype and SMN $\Delta$ 7 mice to perform transcriptional profiling of differentially vulnerable neuron populations (Nichterwitz et al., 2020). This study utilised neuron populations at pre-, early- and late-symptomatic time points to perform a longitudinal study of temporal mechanisms of selective vulnerability in the SMN $\Delta$ 7 mouse model of SMA. By performing gene ontology and protein network analysis, they revealed unique disease-adaptation mechanisms that seem to be present in resistant ocular motor neurons and that could be responsible for their selective sparing (Nichterwitz et al., 2020). These resistant ocular motor neurons were found to upregulate transcripts that are involved in modulation of neurotransmitter release, neuronal survival, and protection from oxidative stress. This study was able to reveal cell specific changes that present promising neuroprotective targets for vulnerable motor neuron populations (Nichterwitz et al., 2020).

All of the above-described studies were able to identify promising modifiers of disease in ALS and SMA by conducting transcriptional analysis of differentially vulnerable motor neuron populations in a single disease model. However, combining transcriptional screens on differentially vulnerable motor neuron populations across different ALS and SMA disease models, can refine the list of potential modifiers and help prioritise those worthy of further validation. This was demonstrated by Kline et al. where a list of thousands of transcripts was refined to 6, with two out of six (SNCA and STMN1) so far being validated as neuroprotective in mouse models of SMA (Kline et al., 2017; Villalón et al., 2019). In this study, they utilised the transcriptome of motor neuron populations, which are differentially vulnerable in SMA, from neurologically normal mice and compared it to three previously published transcriptomes on motor neuron populations, which are differentially vulnerable in ALS, from neurologically normal rats (Hedlund et al., 2010), mice (Kaplan et al., 2014) and human control subjects (Brockington et al., 2013). These findings prove

that combining findings from different disease models and comparisons of differentially vulnerable motor neuron populations increases the likelihood of effective identification of promising modifiers of disease.

In chapter 3 of this thesis, I investigated and compared the selective vulnerability pattern present in the four most commonly used mouse models of SMA. I found that the head (cranial) muscles in the SMN $\Delta$ 7 mouse model of SMA were highly vulnerable (see Chapter 3, Figure 3.9). In contrast, as shown in previously published studies, I found that the head muscles in the *Smn*<sup>2B/-</sup> mouse model are highly resistant to degeneration in SMA (Murray et al., 2015, 2013). These vast differences in the selective vulnerability of the same motor neuron population between the two mouse models was surprising but could be rooted in the differences in their underlying genetics. The severe SMN $\Delta$ 7 model is based on a null mutation of murine *Smn* with two inserted copies of the human *SMN2* transgene, while in the intermediate *Smn*<sup>2B/-</sup> mouse model, murine *Smn* is preserved and mutated to produce lower levels of full-length Smn protein (around 15%) without the need for the induction of human transgenes. These differences in genetics also result in differences in lifespan of these mouse models. While the SMN $\Delta$ 7 model has a lifespan of around 13 days, the *Smn*<sup>2B/-</sup> mouse model, in our hands, has a lifespan of 18 days. The observed sparing of the CN VII motor neurons in the intermediate *Smn*<sup>2B/-</sup> mouse model of SMA, led me to hypothesise that transcripts within this motor neuron population must confer motor neuron protection. Previous studies have utilised resistant CN VII motor neurons in *Smn*<sup>2B/-</sup> mice or vulnerable CN VII motor neurons in SMN $\Delta$ 7 mice for transcriptional analysis in a single disease model to identify potential neuroprotective modifiers (Murray et al., 2015; Nichterwitz et al., 2020). As shown in previous work, the combination of different disease models can facilitate the identification of a refined list of potential modifiers (Kline et al., 2017). I therefore hypothesised that performing a cross-model transcriptional analysis on the CN VII motor neurons from SMA and control animals in both SMN $\Delta$ 7 and *Smn*<sup>2B/-</sup> mouse models, could identify a refined list of modifiers that regulate selective vulnerability in mouse models of SMA, by identifying a transcriptional signature unique to the resistant motor neurons in the *Smn*<sup>2B/-</sup> CN VII nucleus.

Previous studies comparing transcriptomes from differentially vulnerable motor neuron populations in ALS and SMA models constituted the basis for a second cross-model transcriptional analysis in this chapter. Nichterwitz et al. has utilised differentially vulnerable motor neuron populations (CN III/CN IV and CN XII as resistant, and spinal cord as vulnerable) in the SMN $\Delta$ 7 mouse model, while Murray et al. utilised differentially vulnerable motor neurons (CN VII as resistant, spinal cord as vulnerable) from the *Smn*<sup>2B/-</sup> mouse model (Murray et al., 2015; Nichterwitz et al., 2020). Both studies conducted a transcriptional analysis by comparing between SMA and control littermates in a single mouse model of SMA and identified promising modifiers of disease. However, several studies in ALS and SMA showed that very promising neuroprotective modifiers could be identified when utilising screens from only neurologically normal mice and rat littermates from disease models (Hedlund et al., 2010; Kaplan et al., 2014; Kline et al., 2017; Nizzardo et al., 2020). These findings constitute the basis for the second transcriptional analysis conducted in this chapter. Here, I hypothesised that a cross-model transcriptional analysis of differentially vulnerable motor neuron populations from neurologically normal control mice from two SMA mouse models, will yield a refined list of potential neuroprotective modifiers of disease. Motor neuron populations were compared at a time point coinciding with the onset of motor neuron pathology in the SMA models, specifically at P2 in the SMN $\Delta$ 7 and at P10 in the *Smn*<sup>2B/-</sup> mouse model. The identified transcripts have the potential to be utilised for new therapeutic strategies, including both stand-alone, and combinational approaches.

In this chapter of this thesis, I aimed to perform two distinct transcriptional analysis approaches of differentially vulnerable motor neuron populations in two mouse models of SMA. The goal of this work is to identify potential neuroprotective modifiers that have therapeutic potential in SMA mouse models. In this body of work, I first utilised two previously published RNA-sequencing data sets of differentially vulnerable CN VII motor neurons from the SMN $\Delta$ 7 and the *Smn*<sup>2B/-</sup> mouse model of SMA to identify a transcriptional signature unique to the resistant motor neurons in the *Smn*<sup>2B/-</sup> mouse model facial (CN VII) nucleus at a pre-symptomatic time point. I was able to identify

that groups of transcripts associated with protein localisation to cell periphery and ribonucleoprotein complex biogenesis correlated with protection from degeneration. Further, transcriptional analysis on differentially vulnerable motor neurons was conducted on previously published data sets from neurologically normal animals of the SMN $\Delta$ 7 and the *Smn*<sup>2B/-</sup> mouse model of SMA, aiming to identify transcripts which show consistent changes in wildtype motor neurons in populations of cells which are known to be vulnerable in SMA. Results showed a refined list of potential modifiers of motor neuron protection, including PACAP. I further investigated the neuroprotective potential of PACAP in the *Smn*<sup>2B/-</sup> mouse model of SMA. I treated *Smn*<sup>2B/-</sup> mice and their control littermates with PACAP, using IP injections of purified PACAP27 or PACAP38 protein or a single ICV injection of AAV9-PACAP to determine its effect on phenotype and neuropathology. Collectively, data presented in this chapter provides a cross-model comparison of the transcriptional changes occurring in differently vulnerable motor neurons in healthy and SMA animals, identifying and testing potential neuroprotective modifiers of SMA.

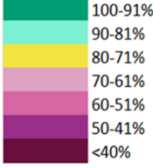
### 4.3. Results

#### 4.3.1. Cross-model transcriptional analysis of CN VII motor neurons reveals groups of transcripts that could contribute to motor neuron protection

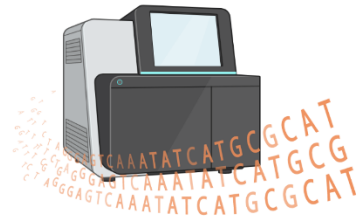
In this chapter, I aimed to conduct a cross-model transcriptional analysis of differentially vulnerable motor neuron populations from the CN VII nucleus from SMN $\Delta$ 7 and the *Smn*<sup>2B/-</sup> mouse model of SMA. The goal was to identify a transcriptional signature unique to the resistant CN VII motor neurons in the *Smn*<sup>2B/-</sup> mouse model at a pre-symptomatic time point. The strategy for this cross-model RNA-sequencing comparison included several steps (Figure 4.1). Raw data was obtained for re-analysis from previously sequenced data sets from differentially vulnerable motor neuron populations from the *Smn*<sup>2B/-</sup> and the SMN $\Delta$ 7 mouse model. I cross-compared data sets of vulnerable CN VII motor neurons from SMN $\Delta$ 7 mice compared to their controls with resistant CN VII motor neurons from *Smn*<sup>2B/-</sup> mice compared to their controls at a pre-symptomatic time-point (P10 in the *Smn*<sup>2B/-</sup> and P2 in the SMN $\Delta$ 7 mouse model). This cross-comparison of two SMA mouse models aimed to identify a transcriptional signature that is unique to the resistant CN VII motor neurons in the *Smn*<sup>2B/-</sup> mouse model. Transcriptional analysis was performed with the primary focus of identifying groups of transcripts in the CN VII of the *Smn*<sup>2B/-</sup> mouse model that could be responsible for the unique sparing of this neuron population. I subsequently used two other comparisons of vulnerable motor neuron population (vulnerable CN VII neurons in SMN $\Delta$ 7 model and vulnerable thoracic spinal cord [tSC] neurons in *Smn*<sup>2B/-</sup> mice) to subtract those from the findings in the resistant *Smn*<sup>2B/-</sup> CN VII motor neurons. These further comparisons of vulnerable motor neuron populations contained changes which are related to the reduction in *Smn* levels and are not associated with protection. The subtraction of these comparisons from the initial resistant *Smn*<sup>2B/-</sup> CN VII motor neurons screen, helped refine a list of transcripts within the *Smn*<sup>2B/-</sup> CN VII motor neurons that might confer protection.

### Identification of target MN populations

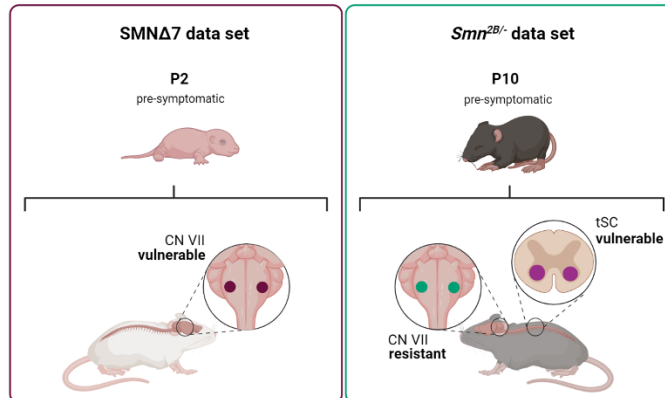
| Muscle Name                         | SMNΔ7 | <i>Smn</i> <sup>2B/-</sup> |
|-------------------------------------|-------|----------------------------|
| Adductor auris longus (AAL)         |       |                            |
| Auricularis superior (AS)           |       |                            |
| Interscutularis (IS)                |       |                            |
| Levator auris longus caudal (LALc)  |       |                            |
| Levator auris longus rostral (LALr) |       |                            |
| Digastric posterior                 |       |                            |
| Masseter                            |       |                            |



### Pre-sequenced data set collection



### MN populations and time points



### RNA-sequencing data analysis

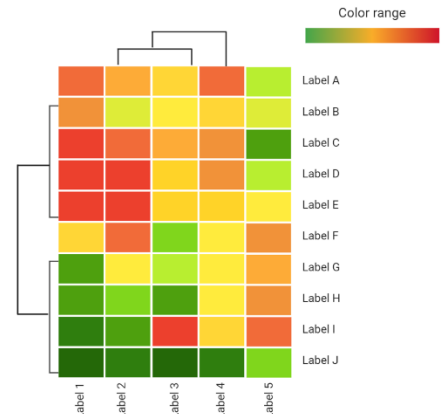


**Figure 4.1: Workflow and strategy of the cross-model RNA-sequencing comparison of CN VII motor neurons.** Motor neuron populations of interest were identified in the SMNΔ7 and *Smn*<sup>2B/-</sup> mouse model of SMA. Raw data was obtained from previously sequenced data sets for re-analysis as cross-model comparison (Murray et al., 2015; Nichterwitz et al., 2020). RNA-sequencing data from a pre-symptomatic time point P2 from SMNΔ7 mice and their control littermates and data from a pre-symptomatic time point P10 from *Smn*<sup>2B/-</sup> mice and their control littermates was utilised for the comparison. In the SMNΔ7 model, motor neurons from the cranial nerve VII nucleus (CN VII), which shows mixed vulnerability, but can overall be classified as vulnerable, were used. In the *Smn*<sup>2B/-</sup> mouse model resistant motor neurons from the CN VII and vulnerable motor neurons from the thoracic spinal cord (tSC) were utilised. Differential gene expression and further transcriptional analysis was performed to identify possible neuroprotective modifiers.

To identify a transcriptional signature unique to the resistant CN VII motor neurons in the *Smn*<sup>2B/-</sup> mouse model of SMA, a list of differentially expressed genes (DEGs) was compiled for each of the three motor neuron populations (resistant CN VII and vulnerable tSC neurons from the *Smn*<sup>2B/-</sup> model and vulnerable CN VII neurons from the SMNΔ7 model). 433 genes were differentially expressed in the resistant CN VII motor neurons when comparing *Smn*<sup>2B/-</sup> mice to their littermate controls. A list of differentially expressed genes in the vulnerable tSC motor neurons from the *Smn*<sup>2B/-</sup> mice showed that 135 genes were differentially expressed when comparing to littermate controls. 58 genes were differentially expressed in the vulnerable CN VII of the SMNΔ7

mouse model when comparing to littermate controls. Next, hierarchical clustering of the 433 DEGs from the resistant CN VII motor neurons in the *Smn*<sup>2B/-</sup> mouse model was performed (Figure 4.2A). The hierarchical clustering on the heatmap clearly indicates a separation between the resistant CN VII motor neurons of the *Smn*<sup>2B/-</sup> mice and the other plotted motor neuron populations. The expression pattern visible in the resistant CN VII *Smn*<sup>2B/-</sup> motor neurons does not match the expression pattern of the vulnerable CN VII SMNΔ7 motor neurons or the patterns of any of the control populations (Figure 4.2A). To identify a transcriptional signature unique to the resistant CN VII motor neurons in the *Smn*<sup>2B/-</sup> mouse model, a list of altered DEGs unique to the resistant *Smn*<sup>2B/-</sup> CN VII data set was compiled. Here, transcripts that were also altered in the *Smn*<sup>2B/-</sup> vulnerable tSC motor neurons (48 transcripts) or SMNΔ7 CN VII motor neurons (1 transcript) compared to their respective controls were removed from the data set (Figure 4.2B). This resulted in the identification of a group of 384 DEGs that was unique to the resistant *Smn*<sup>2B/-</sup> CN VII motor neuron population (Figure 4.2B). To be able to interpret the biological processes that are altered specifically in the *Smn*<sup>2B/-</sup> CN VII motor neurons, the 384 DEGs unique transcripts were used to conduct a Gene Ontology (GO) term enrichment analysis (Fig. 4.2C). The enrichment analysis identified 15 functional clusters for the 384 unique DEGs. The two clusters with the highest gene ratio and the most associated transcripts per cluster were the 'protein localisation to cell periphery' and 'ribonucleoprotein complex biogenesis' clusters. The 'ribonucleoprotein complex biogenesis' GO term cluster (GO:0022613) included 16 transcripts and had an adjusted p-value of p=0.004. In this GO term, genes were associated with cell signalling, rRNA processing and maturation, and ribosome constituents and biogenesis (Figure 4.3A). The GO term cluster 'protein localisation to cell periphery' (GO:1904375) also included 16 differentially expressed genes and had an adjusted p-value of p= 0.002. Genes in this cluster were associated with vesicle trafficking, microtubule dynamics, neurite outgrowth and neuronal development (Figure 4.3B).

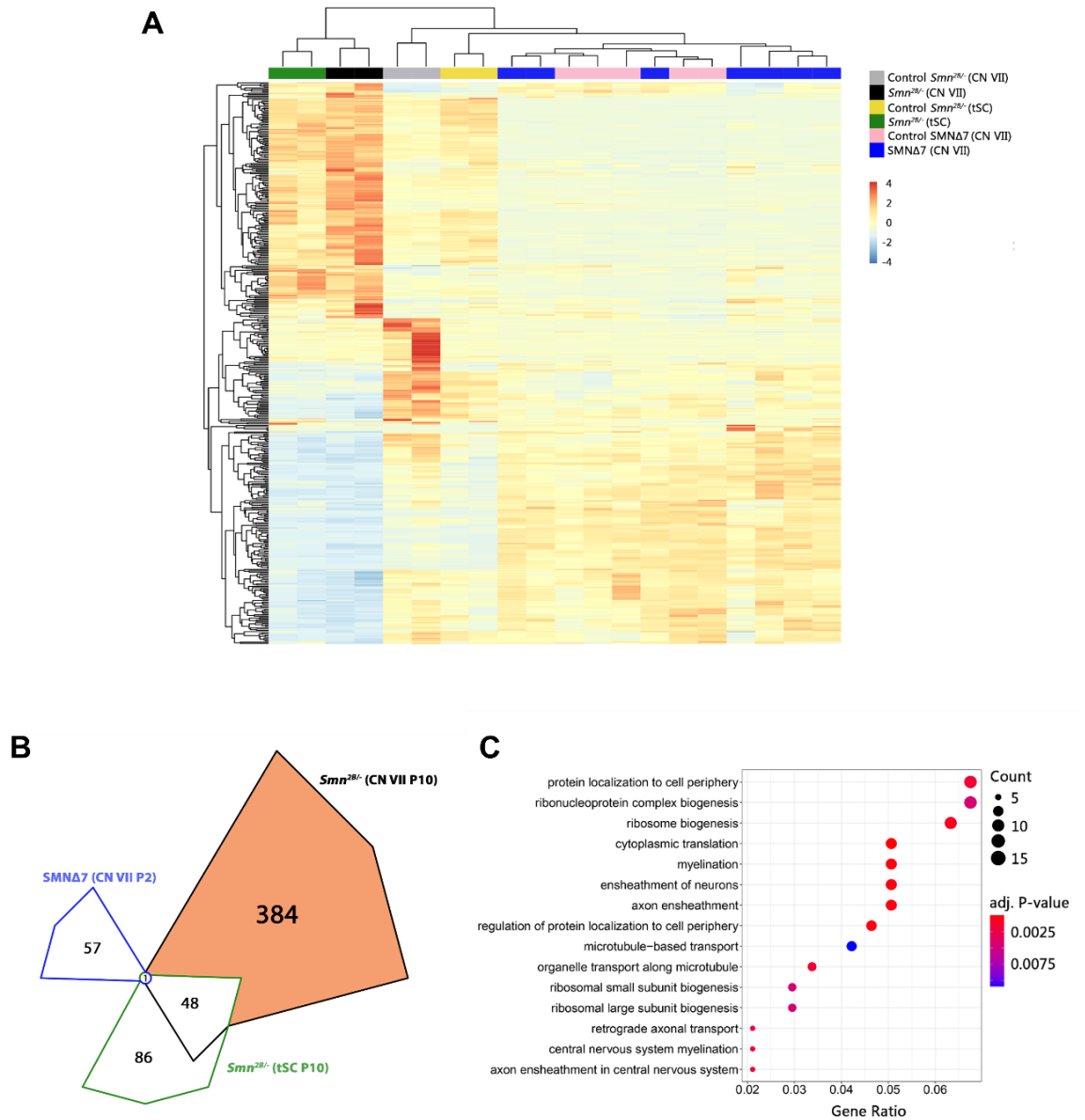


**Figure 4.2: Differential expression analysis across three data sets from SMA mouse models reveals significantly altered transcripts and their biological functions.** **(A)** Expression heatmap of the 433 DEGs between motor neurons from the resistant CN VII in *Smn*<sup>2B/-</sup> mouse model. Relative expression levels in the vulnerable thoracic spinal cord in *Smn*<sup>2B/-</sup> mice and the vulnerable CN VII in SMNΔ7 compared to their respective controls are also shown. Expression values were normalized and mean centred. **(B)** Venn diagram showing the number of overlapping identified DEGs in all three motor neuron groups, showing that 384 DEGs are unique to the resistant CN VII motor neurons in the *Smn*<sup>2B/-</sup> mouse model. **(C)** Gene ontology (GO) term analysis for biological functions of the 384 differentially expressed genes in the CN VII of *Smn*<sup>2B/-</sup> mice compared to control mice. Gene ontology analysis was performed on changes found exclusively in the CN VII motor neurons from *Smn*<sup>2B/-</sup> mice (384 DEGs). The size of the dot in the diagram reflects the number of genes included in the respective GO term. The colour of the dot shows the significance of the adjusted p-value.

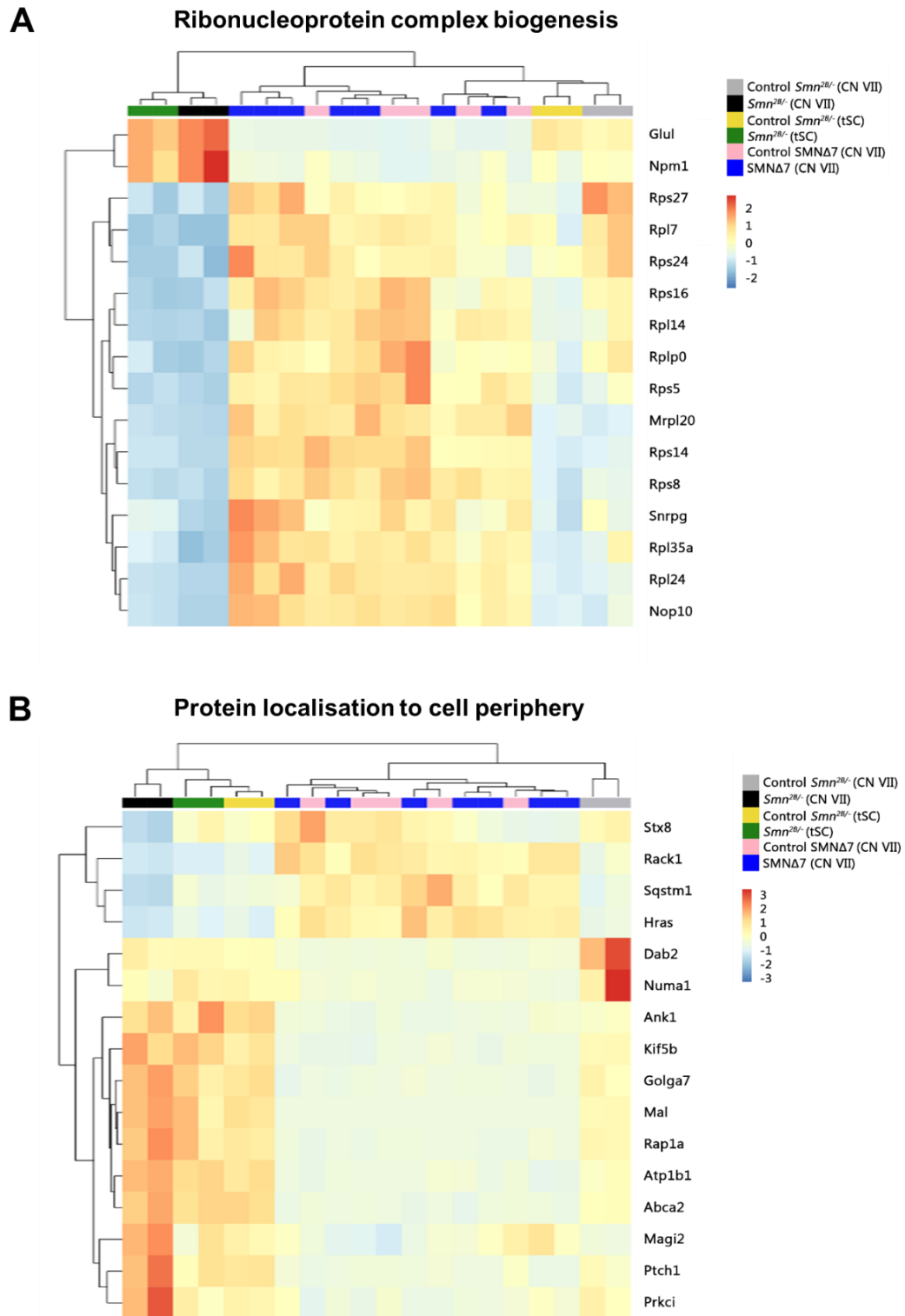


**Figure 4.3: Transcriptional analysis of cross-model comparison shows enriched genes associated with rRNA processing, and vesicle trafficking and neurite outgrowth.** Heatmaps with hierarchical clustering of two significant Gene Ontology (GO) terms, comparing expression between resistant CN VII motor neurons in the *Smn*<sup>2B/-</sup> mouse model and vulnerable CN VII motor neurons in the SMNΔ7 mouse model. Heatmaps show the 16 genes that are included in the GO terms ‘ribonucleoprotein complex biogenesis’ (A), and the 16 genes associated with the GO term cluster ‘protein localisation to cell periphery’ (B). In both heatmaps, z-scores are calculated and plotted instead of the normalised expression value only. Note that in both heatmaps the *Smn*<sup>2B/-</sup> CN VII samples are altered in comparison to their controls, while there is no difference in expression between SMNΔ7 and their controls.

To visualise the interactions between the transcripts in the above-discussed GO term clusters and other dysregulated transcripts, STRING analysis was performed to retrieve networks of protein-protein interactions from all DEGs of *Smn*<sup>2B/-</sup> CN VII samples at P10 (Figure 4.4). Consistent with the GO term analysis that showed a functional cluster of ribonucleoproteins, the STRING analysis revealed that ribosomal proteins from the small subunit (Rps proteins) and the large subunit (Rpl proteins) formed a dense network. Most of the genes in this network (shown in blue in Figure 4.4) contribute to ribosome biogenesis, including *Rps5*, *Rps16*, *Rpl24*, *Rps14* and *Nop10*. Other genes like *Rps27*, *Rps24* and *Snrpg* contribute to pre-rRNA and rRNA processing and maturation. Further, the STRING analysis showed that some genes associated with the 'protein localisation to cell periphery' functional cluster are also part of the protein-protein network interactions (shown in red in Figure 4.4). DEGs among this cluster are important for neuronal development and neurite outgrowth, including *Magi2*, *Kif5b* and *Rap1a*. Other genes like *Numa1*, *Kif5b* and *Prkci* contribute to microtubule dynamics. The network also included *Sqstm1*, which is part of an apoptosis-related network and that is connected to frontotemporal dementia and amyotrophic lateral sclerosis. Lastly, *Snrpg*, which was also included in the functional cluster associated with protein localisation, plays a role in RNA splicing as component of the SMN spliceosome assembly.

In summary, this cross-model transcriptional analysis of differentially vulnerable motor neuron populations in the SMN $\Delta$ 7 and *Smn*<sup>2B/-</sup> mouse models of SMA, identified several transcriptional programs that are specifically activated in the resistant CN VII motor neurons from *Smn*<sup>2B/-</sup> animals. The identified functional clusters of transcripts have the potential to contribute to the protection of motor neurons in the *Smn*<sup>2B/-</sup> mouse models. Further work is needed to functionally validate the neuroprotective potential of these transcripts and networks.

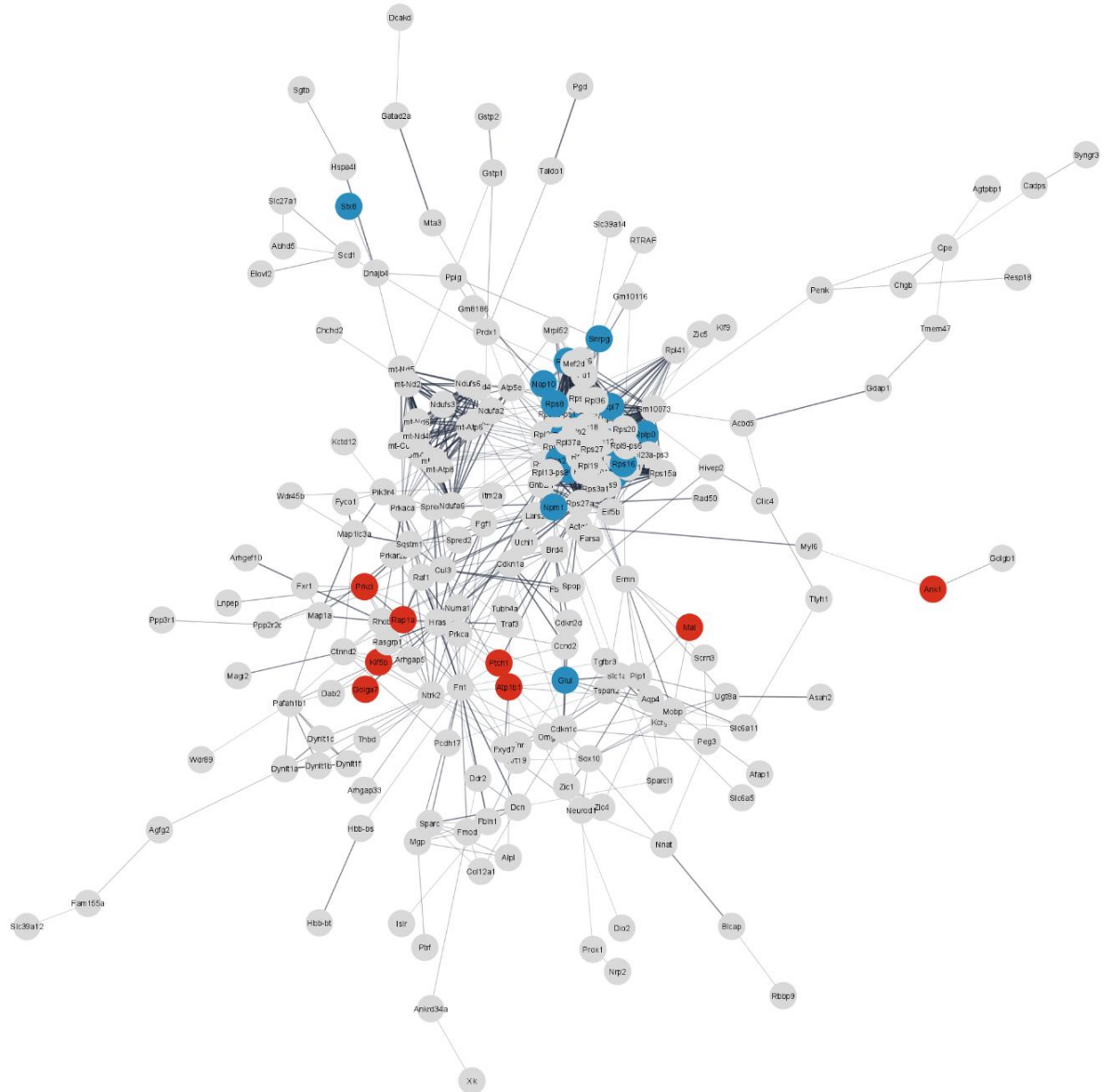


**Figure 4.4: STRING analysis of differentially expressed genes from the *Smn*<sup>2B/-</sup> mouse model reveals a large protein-protein interaction network.** The depicted STRING analysis for protein-protein interactions includes all differentially expressed genes regulated in the CN VII of the *Smn*<sup>2B/-</sup> mouse model of SMA compared to control littermates (384 DEGs). Nodes of the STRING analysis are coloured according to the selected GO terms: ‘protein localisation to cell periphery’ nodes are coloured in red, while nodes associated with ‘ribonucleoprotein complex biogenesis’ are coloured in blue. Isolated nodes without any connections were removed. Network edges represent the confidence of the predicted associations between nodes (edge thickness = strength of data support).

#### **4.3.2. Cross-model transcriptional analysis of differentially vulnerable motor neurons from wildtype mice reveals refined list of potential neuroprotective modifiers**

In a distinct second comparison, I aimed to conduct a transcriptional analysis to identify transcripts which show consistent changes in wildtype motor neurons in populations of cells which are known to be vulnerable in SMA mouse models. Following the hypothesis that differential vulnerability of motor neurons is due to underlying differences in the cell physiology, which will be evident at the transcriptional level, this analysis was performed on motor neuron populations from neurologically normal wildtype (WT) animals only (here control littermates from respective SMA mouse models). The analysis did not include any data from SMA animals. After identifying wildtype motor neuron populations of interest in the SMN $\Delta$ 7 and *Smn*<sup>2B/-</sup> mouse model of SMA, I obtained raw data from the Murray and Nichterwitz data set at comparable time points (P10 in Murray data set [*Smn*<sup>2B/-</sup>] and P2 in Nichterwitz data set [SMN $\Delta$ 7]). These time points coincide with the onset of motor neuron degeneration in the respective SMA mouse models, thereby enabling a comparison of wildtype control mice at a time point where their respective SMA littermates would be at the same stage of disease, before motor neuron pathology manifests and affects the cellular physiology. Three motor neuron populations, which are differentially vulnerable in the SMN $\Delta$ 7 mouse model (Nichterwitz data set), were used from wildtype mice: the resistant cranial nerve nucleus CN III/ CN IV, the resistant CN XII, and the vulnerable spinal cord (SC) (Figure 4.5A). Two motor neuron populations, which are differentially vulnerable in the *Smn*<sup>2B/-</sup> mouse model (Murray data set), were utilised from wildtype mice: the resistant CN VII motor neurons and the vulnerable spinal cord (SC) motor neurons (Figure 4.5A). Comparative transcriptional analysis was performed in three stages to identify a refined list of possible neuroprotective modifiers (Figure 4.5B). In the first two stages of this analysis, a population of motor neurons which is vulnerable in the Nichterwitz data set (SC, depicted in blue rectangle in Figure 4.5) and one that is resistant in the Nichterwitz data set (CN III/CN IV or CN XII, depicted in blue rectangle in Figure 4.5) were examined in wildtype mice. Similarly, a population of motor neurons which is vulnerable in the Murray data set (SC, depicted in yellow

rectangle in Figure 4.5) and one that is resistant in the Murray data set (CN VII, depicted in yellow rectangle in Figure 4.5) were examined in wildtype mice. These results were then cross-compared between the Nichterwitz and Murray data set, thereby producing lists of transcripts that were common in wildtype motor neurons between the two data sets. Subsequently, in a third comparison, the two produced lists of common transcripts were cross-compared, and transcripts that were common across both initial lists were identified as refined list of potential modifiers (Figure 4.5B).

For the first stage of this transcriptional analysis, differential expression analysis was performed on wildtype resistant CN III/CN IV motor neurons compared to vulnerable SC motor neurons in the Nichterwitz data set. Further, differentially expressed genes were identified by comparing wildtype resistant CN VII and vulnerable SC motor neurons in the Murray data set. Cross-comparison of the results of both RNA-sequencing comparisons (DEGs in Nichterwitz and DEGs in Murray data set) revealed 1111 commonly changed transcripts shared between both data sets. For the identification of a refined list of modifiers, particular interest was shown for altered transcripts that displayed the same directional change in both data sets. The majority of commonly changed transcripts in this analysis showed the same directional change, identifying 557 altered transcripts that are shared between wildtype mice in Nichterwitz and Murray data sets. Functional clustering of the 557 identified altered transcripts was performed using DAVID bioinformatics resource (Figure 4.6A). 91 functional clusters were found, of which 19 functional clusters were significant with an enrichment score of  $>1.3$  (equalling a p-value of  $p < 0.05$ ). This analysis revealed an enrichment of transcripts associated with synapse and extracellular matrix (Figure 4.6A). Of the 557 identified commonly altered transcripts between wildtype motor neurons from both data sets, 79 transcripts were significantly altered with a  $\log_2$  fold change of  $>1.5$  fold and a p-value of  $p < 0.05$ . 22 of these transcripts were up-regulated (Figure 4.6B), while 57 transcripts were down-regulated (Figure 4.6C) in vulnerable versus resistant motor neurons.

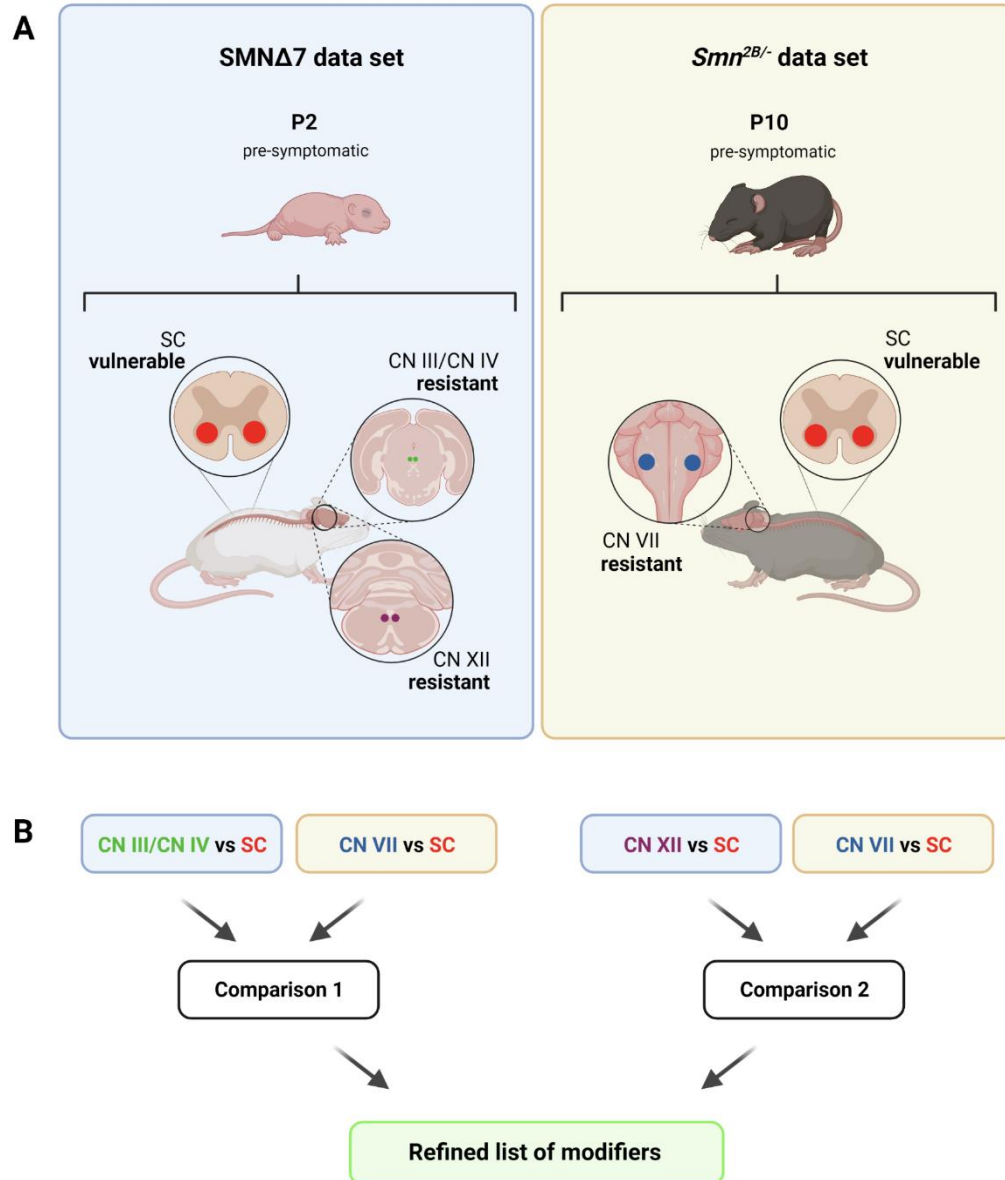


**Figure 4.5: Strategy of the RNA-sequencing comparison of wildtype motor neurons from two data sets. (A)** Raw data was obtained from previously sequenced data sets for re-analysing as cross-model comparison (Murray et al., 2015; Nichterwitz et al., 2020). RNA-sequencing data from wildtype mice at time point P2 (SMNΔ7 control littermates) from Nichterwitz data set and data from wildtype mice at time point P10 (*Smn*<sup>2B/-</sup> control littermates) from Murray data set was utilised for the comparison. In the Nichterwitz data set, motor neurons from the vulnerable spinal cord (SC) and the resistant cranial nerve nuclei CN III/CN IV and CN XII were used. In the Murray data set resistant motor neurons from the CN VII and vulnerable motor neurons from the spinal cord were utilised. **(B)** Comparison strategy of the cross-model comparison of wildtype motor neurons from Nichterwitz and Murray data sets to yield a refined list of potential neuroprotective modifiers. In the first two comparisons a resistant and a vulnerable motor neuron population from wildtype motor neurons were compared within the Nichterwitz data set and within the Murray data set (Nichterwitz depicted in blue, Murray in yellow, motor neuron population coloured according to diagram in panel A). These results were cross-compared between the Nichterwitz and Murray data sets, thereby producing lists of transcripts that were common in wildtype motor neurons between the two data sets. Subsequently, in a third comparison, the two produced lists of common transcripts were cross-compared, and transcripts that were common across both initial lists were identified as refined list of potential modifiers.

| <b>A</b> | <b>Functional Cluster Terms</b>                   | <b>Enrichment Score</b> |
|----------|---------------------------------------------------|-------------------------|
|          | Synapse, cell junction                            | 5.9                     |
|          | Extracellular matrix                              | 3.7                     |
|          | Palmitate, lipoprotein                            | 3.2                     |
|          | Neurogenesis, differentiation                     | 2.5                     |
|          | Ion transport, ion channel,                       | 2.4                     |
|          | Homeobox                                          | 2.3                     |
|          | Neurotransmitter symporter, synaptic transmission | 2.3                     |
|          | Metal-binding, zinc, zinc-finger                  | 2.2                     |
|          | Differentiation, developmental protein            | 2.1                     |
|          | Collagen, hydroxylation                           | 1.9                     |

| <b>B</b> | <b>Gene Symbol</b> | <b>log<sub>2</sub> fold change</b> |             |
|----------|--------------------|------------------------------------|-------------|
|          |                    | Murray                             | Nichterwitz |
|          | Hoxc8              | 11.1                               | 6.2         |
|          | Hoxd8              | 10.4                               | 5.4         |
|          | Hoxc9              | 7.8                                | 6.0         |
|          | Hoxa5              | 7.1                                | 6.2         |
|          | Npy5r              | 6.8                                | 2.0         |
|          | Gfra1              | 6.5                                | 1.8         |
|          | Egln3              | 6.3                                | 2.2         |
|          | Slc6a5             | 5.6                                | 2.0         |
|          | Hoxd9              | 5.5                                | 5.4         |
|          | Hoxb7              | 5.5                                | 3.6         |

| <b>C</b> | <b>Gene Symbol</b> | <b>log<sub>2</sub> fold change</b> |             |
|----------|--------------------|------------------------------------|-------------|
|          |                    | Murray                             | Nichterwitz |
|          | Slc6a4             | -9.6                               | -2.4        |
|          | Tph2               | -9.4                               | -1.6        |
|          | Tbx20              | -8.3                               | -6.6        |
|          | Ranbp3l            | -7.1                               | -1.8        |
|          | Col5a2             | -6.4                               | -3.9        |
|          | Irx1               | -6.2                               | -3.8        |
|          | Efemp1             | -5.9                               | -2.4        |
|          | Prg4               | -5.9                               | -2.1        |
|          | A930019D19Rik      | -5.1                               | -2.1        |
|          | Pou4f1             | -5.1                               | -2.5        |

**Figure 4.6: Functional clustering and altered transcripts in wildtype cross-model transcriptional analysis (Nichterwitz: CN III/CN IV vs SC and Murray: CN VII vs SC).** Wildtype motor neuron populations from the Nichterwitz and Murray data set were compared. In the Nichterwitz data set, wildtype motor neurons from the vulnerable spinal cord (SC) and the resistant CN III/CN IV were compared at time point P2. In the Murray data set, wildtype resistant motor neurons from the CN VII and vulnerable motor neurons from the spinal cord were compared at a time point P10. **(A)** Table showing the top 10 functional clusters of transcriptional changes between motor neurons from the Nichterwitz (CN III/CN IV vs SC) and Murray (CN VII vs SC) data sets. Functional clusters were identified using DAVID functional clustering. **(B-C)** Tables showing the top 10 significantly up- and down regulated transcripts identified between motor neurons from the Nichterwitz (CN III/CN IV vs SC) and Murray (CN VII vs SC) data sets respectively. The tables display the log<sub>2</sub> fold change in expression from both data sets for each transcript. Order of transcripts is reflective of the log<sub>2</sub> fold change in the Murray data set. Genes were considered significantly differentially expressed with a p-value of  $p \leq 0.05$  and a log<sub>2</sub> fold change in expression greater than  $\pm 1.5$ .

For the second stage of this transcriptional analysis, differentially vulnerable motor neuron populations from wildtype motor neurons from the Nichterwitz and Murray data set were again compared at a time point coinciding with the onset of motor neuron pathology in the SMA models (P10 in the *Smn*<sup>2B/-</sup> and P2 in the SMN $\Delta$ 7 mouse model). Differential expression analysis was performed on the same wildtype cells from the Murray data set as in the first stage of the transcriptional comparison (resistant CN VII versus vulnerable SC). However, for the differential expression analysis of tissues from the Nichterwitz data set, the vulnerable SC wildtype motor neuron population remained the same, while a different resistant wildtype motor neuron population was chosen for comparison: the cranial nerve nucleus XII (CN XII). After differentially expressed genes were identified in both mouse models (CN VII vs SC in Murray data set and CN XII vs SC in Nichterwitz data set), cross-comparison of the results of both RNA-sequencing comparisons revealed 1132 commonly changed transcripts shared between both data sets. Again, the majority of commonly changed transcripts in this analysis showed the same directional change, identifying 599 altered transcripts that are shared between wildtype mice in Nichterwitz and Murray data sets. Functional clustering using DAVID bioinformatics resource was performed on the 599 identified altered transcripts (Figure 4.7A). 108 functional clusters were identified, of which 26 functional clusters were significant with an enrichment score of >1.3 (equalling a p-value of p<0.05), and revealing an enrichment associated with extracellular matrix and glycoprotein (Figure 4.7A). Of the 599 transcripts with the same directional change between wildtype motor neurons in both data sets, 95 transcripts were significantly altered with a log<sub>2</sub> fold change of >1.5 fold and a p-value of p<0.05. Of these transcripts, 23 were up-regulated (Figure 4.7B) and 72 transcripts were down-regulated (Figure 4.7C) in vulnerable versus resistant motor neurons.

| <b>A</b> | <b>Functional Cluster Terms</b>      | <b>Enrichment Score</b> |
|----------|--------------------------------------|-------------------------|
|          | Extracellular matrix                 | 11.5                    |
|          | Glycoprotein, signal, secreted       | 6.6                     |
|          | Synapse, cell junction               | 4.9                     |
|          | Collagen, hydroxylation              | 3.8                     |
|          | Palmitate, lipoprotein               | 3.3                     |
|          | VWFA (von Willebrand factor, type A) | 2.3                     |
|          | Synaptic vesicle cycle               | 2.2                     |
|          | Amino-acid transport                 | 2.0                     |
|          | NADP                                 | 1.9                     |
|          | Behaviour                            | 1.8                     |

| <b>B</b> | <b>Gene Symbol</b> | <b>log<sub>2</sub> fold change</b> |             |
|----------|--------------------|------------------------------------|-------------|
|          |                    | Murray                             | Nichterwitz |
|          | Hoxc8              | 11.1                               | 7.0         |
|          | Hoxd8              | 10.4                               | 5.4         |
|          | Hoxc9              | 7.8                                | 6.0         |
|          | Hoxd9              | 6.6                                | 5.4         |
|          | Hoxb7              | 5.5                                | 3.7         |
|          | Frem3              | 4.4                                | 3.9         |
|          | Gm10710            | 4.1                                | 3.0         |
|          | A730020M07Rik      | 4.0                                | 3.4         |
|          | Mybpc1             | 3.9                                | 3.4         |
|          | Adamtsl2           | 3.9                                | 1.5         |

| <b>C</b> | <b>Gene Symbol</b> | <b>log<sub>2</sub> fold change</b> |             |
|----------|--------------------|------------------------------------|-------------|
|          |                    | Murray                             | Nichterwitz |
|          | Nov                | -8.5                               | -4.7        |
|          | Gal                | -7.9                               | -2.4        |
|          | Thbd               | -7.5                               | -1.6        |
|          | Islr               | -5.9                               | -2.1        |
|          | Adcyap1            | -5.8                               | -2.9        |
|          | Col5a2             | -5.6                               | -3.9        |
|          | Lrrk1              | -5.6                               | -2.2        |
|          | Vip                | -5.4                               | -4.9        |
|          | Shisa3             | -5.3                               | -4.3        |
|          | Lox                | -5.3                               | -4.8        |

**Figure 4.7: Functional clustering and altered transcripts in wildtype cross-model transcriptional analysis (Nichterwitz: CN XII vs SC and Murray: CN VII vs SC).** Wildtype motor neuron populations from the Nichterwitz and Murray data sets were compared. In the Nichterwitz data set, wildtype motor neurons from the vulnerable spinal cord (SC) and the resistant CN XII were compared at a time point P2. In the Murray data set, wildtype resistant motor neurons from the CN VII and vulnerable motor neurons from the spinal cord were compared at a time point P10. **(A)** Table showing the top 10 functional clusters of transcriptional changes between motor neurons from the Nichterwitz (CN XII vs SC) and Murray (CN VII vs SC) data sets. Functional clusters were identified using DAVID functional clustering. **(B-C)** Tables showing the top 10 significantly up- and down regulated transcripts identified between motor neurons from the Nichterwitz (CN XII vs SC) and Murray (CN VII vs SC) data sets respectively. The tables display the log<sub>2</sub> fold change in expression from both data sets for each transcript. Order of transcripts is reflective of the log<sub>2</sub> fold change in the Murray data set. Genes were considered significantly differentially expressed with a p-value of p≤0.05 and a log<sub>2</sub> fold change in expression greater than ±1.5.

For the third and final stage of this transcriptional analysis, the produced lists with commonly altered transcripts from the previous two stages of this analysis were compared. The 557 common DEGs from the first stage of the analysis (which included the resistant CN III/CN IV wildtype motor neurons from the Nichterwitz data set) and the 599 common DEGs from the second stage of the analysis (which included the resistant CN XII wildtype motor neurons from the Nichterwitz data set) were compared to each other to produce a refined list of potential neuroprotective modifiers. Cross-comparison of the compiled lists from both previous RNA-sequencing comparisons revealed 331 commonly changed transcripts that showed the same directional change between wildtype mice from all data sets. Functional clustering was performed on the 331 identified DEGs using DAVID bioinformatics resource (Figure 4.8A). 62 functional clusters were found, of which 10 functional clusters were significant with an enrichment score of  $>1.3$  (equalling a p-value of  $p < 0.05$ ). This analysis revealed an enrichment of transcripts associated with synapse, synaptic vesicle membrane, and extracellular matrix (Figure 4.8A). Of the 331 identified commonly altered transcripts of wildtype motor neurons, 25 transcripts were significantly altered with a  $\log_2$  fold change of  $>1.5$  fold and a p-value of  $p < 0.05$ . 15 of these transcripts were up-regulated (Figure 4.8B), while 10 transcripts were down-regulated (Figure 4.8C) in vulnerable versus resistant motor neurons. The 25 significantly altered transcripts identified in this final stage of the transcriptional analysis performed in this chapter, all show a common directional change between the Nichterwitz and Murray data set. These 25 significant DEGs are the result of the cross-comparison between the Nichterwitz data set (two comparisons performed: 1. CN III/CN IV vs SC; 2. CN XII vs SC) and the Murray data set (one comparison performed: CN VII vs SC).

The results of the transcriptional analysis performed in this chapter, show that I was able to produce a refined list of 25 potential modifiers of selective vulnerability in SMA mouse models. The identified refined list yielded promising candidates of neuroprotection, including *Col5a2*, *Gabra1*, *Gfra1*, and *Adcyap1*. Previous studies showed that NMJs present with an impaired function in the absence of a similar collagen to ColIV (Cescon et al., 2018). A

study further indicated *Gabra1* as candidate for oculomotor resistance in ALS mice and patients (Comley et al., 2015), while other work identified the specific requirement of *Gira1* for motor function in zebrafish (Samarut et al., 2019). Lastly, *Adcyap1*, also referred to as PACAP, was proven to offer potent neuroprotection in various neurodegenerative disease models, including in *in vivo* and *in vitro* models of ALS (Bonaventura et al., 2018; D'Amico et al., 2020; Maugeri et al., 2019; Ringer et al., 2013).

In summary, this cross-model transcriptional analysis of wildtype motor neuron populations, which are differentially vulnerable in the *SMNΔ7* and *Smn<sup>2B/-</sup>* mouse models of SMA, identified a refined list of potential neuroprotective modifiers in SMA mouse models. Some of the 25 transcripts identified in this transcriptional analysis, could be promising candidates for *in vivo* testing of their potential neuroprotective properties in mouse models of SMA.

| A |  | Functional Cluster Terms         | Enrichment Score |
|---|--|----------------------------------|------------------|
|   |  | Synapse, cell junction           | 5.3              |
|   |  | Palmitate, lipoprotein           | 3.4              |
|   |  | Metal-binding, zinc, zinc-finger | 2.4              |
|   |  | Syntaxin-binding                 | 2.4              |
|   |  | Synaptic vesicle membrane        | 2.2              |
|   |  | Extracellular matrix             | 2.1              |
|   |  | Cytoplasmic vesicle              | 1.9              |
|   |  | SNARE complex, SNARE binding     | 1.6              |
|   |  | Homeobox                         | 1.5              |
|   |  | Glycoprotein, disulfide bond     | 1.5              |

| B             |  | log <sub>2</sub> fold change |                             |                       |
|---------------|--|------------------------------|-----------------------------|-----------------------|
| Gene Symbol   |  | Murray                       | Nichterwitz<br>CN III/CN IV | Nichterwitz<br>CN XII |
| Hoxc8         |  | 11.1                         | 6.2                         | 7.0                   |
| Hoxd8         |  | 10.4                         | 5.4                         | 5.4                   |
| Hoxc9         |  | 7.8                          | 6.0                         | 6.0                   |
| Hoxd9         |  | 5.4                          | 5.5                         | 6.6                   |
| Gira1         |  | 1.8                          | 6.5                         | 1.9                   |
| Egln3         |  | 2.2                          | 6.3                         | 3.5                   |
| Hoxb7         |  | 5.5                          | 3.6                         | 3.7                   |
| Frem3         |  | 3.9                          | 1.8                         | 4.4                   |
| Gm10710       |  | 3.0                          | 2.5                         | 4.1                   |
| A730020M07Rik |  | 3.4                          | 3.9                         | 4.0                   |

| C           |  | log <sub>2</sub> fold change |                             |                       |
|-------------|--|------------------------------|-----------------------------|-----------------------|
| Gene Symbol |  | Murray                       | Nichterwitz<br>CN III/CN IV | Nichterwitz<br>CN XII |
| Col5a2      |  | -3.9                         | -6.4                        | -5.6                  |
| Adcyap1     |  | -2.9                         | -1.8                        | -5.8                  |
| Pou4f1      |  | -5.1                         | -2.5                        | -2.5                  |
| Dcdc2a      |  | -2.9                         | -3.8                        | -2.0                  |
| Gabra1      |  | -1.8                         | -3.5                        | -1.8                  |
| Rnft1       |  | -2.4                         | -3.4                        | -1.5                  |
| Adamts15    |  | -3.2                         | -1.8                        | -1.7                  |
| AW551984    |  | -1.8                         | -1.5                        | -2.8                  |
| Fbln2       |  | -2.7                         | -2.3                        | -2.1                  |
| Armc2       |  | -2.1                         | -1.7                        | -1.7                  |

**Figure 4.8: Functional clustering and altered transcripts of wildtype cross-model transcriptional analysis yields refined list of potential neuroprotective modifiers.** Lists of common transcripts from wildtype motor neuron populations from the Nichterwitz and Murray data set were compared. Two identified lists of common transcripts between wildtype motor neurons from the Nichterwitz data set and Murray data set were cross-compared. Transcripts that were common across both previously compiled lists were identified as refined list of modifiers. A first comparison yielded a list of common transcripts between motor neurons from the Nichterwitz (CN III/CN IV vs SC) and Murray (CN VII vs SC) data sets, while a second comparison yielded a list of common transcripts between different motor neuron populations from the Nichterwitz (CN XII vs SC) and Murray (CN VII vs SC) data sets. **(A)** Table showing the top 10 functional clusters of transcriptional changes cross-comparing results from two previously identified lists of common transcripts between wildtype motor neurons from the Nichterwitz and Murray data sets. Functional clusters were identified using DAVID functional clustering. **(B-C)** Tables showing the top 10 significantly up- and down regulated transcripts, cross-comparing results from two previously identified lists of common transcripts between wildtype motor neurons from the Nichterwitz and Murray data sets. The tables display the log<sub>2</sub> fold change in expression from all data sets for each transcript. Order of transcripts is reflective of the log<sub>2</sub> fold change in the Murray data set. Genes were considered significantly differentially expressed with a p-value of  $p \leq 0.05$  and a log<sub>2</sub> fold change in expression greater than  $\pm 1.5$ .

#### **4.3.3. Investigating the neuroprotective properties of intraperitoneal PACAP treatment on the phenotype of the *Smn*<sup>2B/-</sup> mouse model**

Following the cross-model transcriptional analysis of wildtype motor neuron populations, which are differentially vulnerable in the SMNΔ7 and *Smn*<sup>2B/-</sup> mouse models of SMA, a refined list of potential neuroprotective modifiers was yielded. Previous studies suggest that some of the identified transcripts from this RNA-sequencing analysis have the potential to be promising modifiers of motor neuron protection in various neurodegenerative diseases. A promising potential modifier that was identified in this chapter of this thesis was *Adcyap1*. *Adcyap1* encodes the pituitary adenylate cyclase-activating polypeptide (PACAP) protein. PACAP is a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family (Arimura and Shioda, 1995). PACAP has two biologically active isoforms that depend on the number of amino acids in the N-terminus: PACAP27 and PACAP38 (Arimura, 1992), where PACAP38 seems to be the predominantly expressed form in the mammalian brain (Vaudry et al., 2009). Neuroprotective effects of exogenous PACAP administration have been demonstrated in various neurodegenerative diseases, including stroke, traumatic brain injury, spinal cord injury, Parkinson's disease, and Huntington's disease (Fang et al., 2020). Previous work further showed neuroprotective properties of PACAP in ALS, demonstrating that PACAP protected against pre-ganglionic visceromotor neuron loss in a mouse model of ALS (Ringer et al., 2013), and that PACAP rescued motor neuron degeneration following growth factor deprivation in iPSCs of ALS (Bonaventura et al., 2018). Studies further showed that PACAP increased viability of cells, decreased apoptosis, and had a positive effect on the autophagy process of cells in a MN-like model of ALS (D'Amico et al., 2020; Maugeri et al., 2019). The widespread neuroprotective properties of PACAP in diverse neurodegenerative diseases, including in various disease models of ALS, coupled with the possibility of a direct exogenous administration of the PACAP protein, makes this modifier the ideal candidate for *in vivo* testing in a mouse model of SMA.

In this chapter, I administered 182.49mg/kg of purified PACAP27 protein, 251.8mg/kg of purified PACAP38 protein or saline as vehicle control via daily

intraperitoneal (IP) injections from P5 until end of life to *Smn*<sup>2B/-</sup> mice and their control littermates, and quantified the effects on weight, motor phenotype and levels of NMJ loss (Figure 4.9A). Since it was previously demonstrated that PACAP38 has a very short half-life *in vitro* (less than 5 minutes in human plasma) but PACAP27 is more stable (Bourgault et al., 2008), I tested both PACAP isoforms: PACAP27 and PACAP38.

Weight curves from all six experimental groups (i.e. *Smn*<sup>2B/-</sup> animals treated with PACAP27, PACAP38 or saline; control animals treated with PACAP27, PACAP38 or saline) showed that there was no significant difference in the weights of *Smn*<sup>2B/-</sup> animals that were treated with PACAP27 (182.49mg/kg), PACAP38 (251.8mg/kg) or saline between P5 and disease end-stage (Figure 4.9B). The same decrease in weight in comparison to control littermates could be observed in all three *Smn*<sup>2B/-</sup> groups. Next, the motor performance of the PACAP and saline treated *Smn*<sup>2B/-</sup> and control animals was evaluated. Motor tests were performed 2 hours after they received their last injection of PACAP27, PACAP38 or saline at disease end-stage on the last day of life (Figure 4.9C). Results showed that there was no significant difference in the motor performance of the PACAP27 or PACAP38 treated *Smn*<sup>2B/-</sup> animals in comparison to the saline treated *Smn*<sup>2B/-</sup> animals (Figure 4.9C). All three treatment groups of *Smn*<sup>2B/-</sup> animals showed a significant increase in time that it took them to perform the motor test in comparison to their controls. The majority of *Smn*<sup>2B/-</sup> animals throughout all treatment groups failed the test, taking 30 seconds or longer to turn around on the grid. These findings suggest that neither IP administered PACAP27, nor PACAP38 have an effect on the motor phenotype of the *Smn*<sup>2B/-</sup> mouse model. Neither isoform of PACAP was able to improve weight gain or motor performance in the *Smn*<sup>2B/-</sup> mice in comparison to their saline treated *Smn*<sup>2B/-</sup> control littermates.



n=5 for PACP38 *Smn*<sup>2B/-</sup> mice, n=8 for PACAP27 and saline *Smn*<sup>2B/-</sup> mice, and n=12 for PACAP27 controls. Error bars represent mean  $\pm$  SEM. **(C)** Bar graph shows how quickly (in seconds) *Smn*<sup>2B/-</sup> mice and control animals completed the time to turn motor test at disease end-stage. Note that there is no significant difference in the motor performance between saline treated *Smn*<sup>2B/-</sup> mice and *Smn*<sup>2B/-</sup> mice that were treated with either isoform of PACAP. One-way ANOVA with Tukey correction (ns= no significance, \*\*p $\leq$ 0.01, \*\*\*p $\leq$ 0.001). n=3 for Saline controls, n=4 for PACP38 controls, n=5 for PACAP27 and PACAP38 *Smn*<sup>2B/-</sup> mice and PACAP27 controls, and n=6 for saline *Smn*<sup>2B/-</sup> mice. Error bars represent mean  $\pm$  SEM.

To investigate if the IP injected PACAP27 (182.49mg/kg) or PACAP38 (251.8mg/kg) was able to reach target tissues after 12 consecutive days of IP administration, the fluorescent intensity levels associated with the FITC tag of the administered PACAP isoforms were analysed (Figure 4.10). As most compounds that are administered through the IP route enter the systemic circulation by passing through the liver (Al Shoyaib et al., 2019), the fluorescent intensity present in liver samples from *Smn*<sup>2B/-</sup> mice treated with PACAP27, PACAP38 or with saline (as control group) were analysed and provided by MSc student Estefania Grados-Porro. Results showed that there was no significant difference in the fluorescent intensity in liver samples between saline treated and PACAP27 or PACAP38 treated *Smn*<sup>2B/-</sup> animals (Figure 4.10A). Next, to determine if IP injected PACAP27 and PACAP38 crossed the blood-brain barrier and therefore were able to reach the central nervous system, the fluorescent intensity levels present in brain samples from *Smn*<sup>2B/-</sup> mice treated with PACAP27, PACAP38 or with saline were analysed and provided by MSc student Estefania Grados-Porro. Results showed that there was no significant difference in the fluorescent intensity in brain samples between saline treated and PACAP27 or PACAP38 treated *Smn*<sup>2B/-</sup> animals (Figure 4.10B). However, a non-significant trend could be seen in the PACAP27 treated animals compared to the saline treated animals, suggesting that the PACAP27 isoform might have crossed the blood-brain barrier and possibly entered the cells.

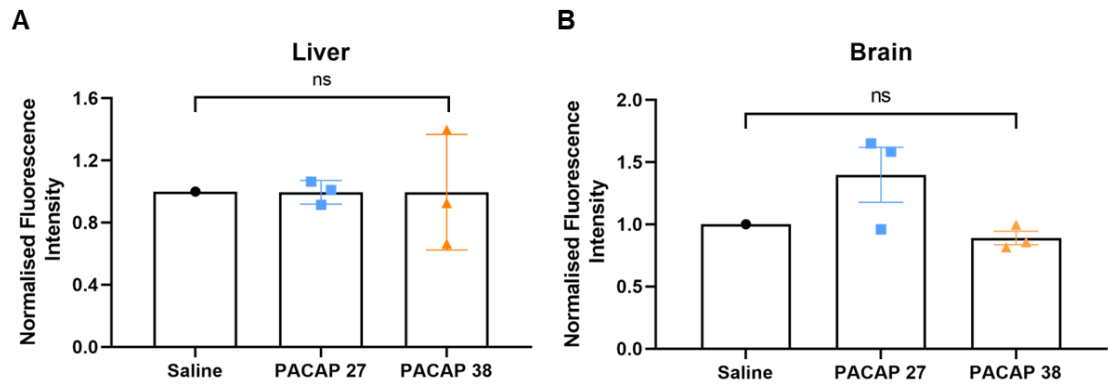


**Figure 4.10: PACAP27 treatment administered as daily intraperitoneal injections shows no significant increase in fluorescence intensity in the *Smn*<sup>2B/-</sup> mouse model at disease end-stage.** (A) Bar chart showing the quantification of normalised fluorescence intensity in liver following daily PACAP27 (182.49mg/kg), PACAP38 (251.8mg/kg) and saline treatment in *Smn*<sup>2B/-</sup> mice. Note that there was no significant difference between PACAP27, PACAP38 and saline treated *Smn*<sup>2B/-</sup> mice. Kruskal-Wallis multiple comparisons test with Dunn's correction (ns = not significant). n=3 samples (4 pictures per sample analysed) for all genotypes. Error bars represent mean  $\pm$  SEM. (B) Bar chart showing the quantification of normalised fluorescence intensity in brain following daily PACAP27, PACAP38 and saline treatment in *Smn*<sup>2B/-</sup> mice. Note that there was no significant difference between PACAP27, PACAP38 and saline treated *Smn*<sup>2B/-</sup> mice. Kruskal-Wallis multiple comparisons test with Dunn's correction (ns = not significant). n=6 for PACAP27, n=3 for PACAP38 and n=5 for saline controls. 4 images per sample were analysed for all n-numbers for all genotypes. Error bars represent mean  $\pm$  SEM.

To further investigate the potential neuroprotective effect of PACAP in the *Smn*<sup>2B/-</sup> mouse model of SMA, the effect of PACAP treatment on levels of NMJ pathology was investigated. The above-presented results showed that there does not seem to be a significant difference in effectiveness between the two PACAP isoforms. Coupled with observations from an *in vitro* study, showing that PACAP27 remained stable with no signs of degradation after 45 minutes, while PACAP38 was rapidly metabolised with a half-life of less than 5 minutes (Bourgault et al., 2008), the following experiments were conducted on *Smn*<sup>2B/-</sup> and control animals treated with PACAP27 or saline (as vehicle control) only. To investigate a potential beneficial effect of PACAP treatment on NMJ pathology in the *Smn*<sup>2B/-</sup> mouse model, I first investigated the level of synaptic loss that was present in the vulnerable TVA muscle (Courtney et al., 2019; Murray et al., 2015, 2013). NMJ occupancy quantification was performed on four genotypes: PACAP27 (182.49mg/kg) and saline treated *Smn*<sup>2B/-</sup> mice, and PACAP27 (182.49mg/kg) and saline treated control littermates. Results showed that there was no significant difference in the remaining fully

innervated endplates between PACAP27 and saline treated *Smn*<sup>2B/-</sup> animals (Figure 4.11B). There was also no significant difference in the number of vacant endplates at disease end-stage between PACAP27 and saline treated *Smn*<sup>2B/-</sup> mice. As expected, there was a significant decrease in the fully innervated endplates between the saline treated *Smn*<sup>2B/-</sup> mice and their control littermates (Figure 4.11B). The same pattern could also be observed in the PACAP27 treated *Smn*<sup>2B/-</sup> mice and their respective control littermates.

Next, the amount of pre-synaptic swelling was quantified in the TVA muscle of the *Smn*<sup>2B/-</sup> mice and their control littermates. The pre-synaptic swelling was classified as one of four stages: none, mild, moderate, or severe. A full description of the criteria of pre-synaptic swelling in immuno-stained muscles can be found in Chapter 2 section 2.5.3. Briefly, pre-synaptic swelling was classified as none, if there was no evidence of pre-synaptic or axonal swelling, as mild if there was some evidence of pre-synaptic or axonal swelling, as moderate if there was clear axonal and pre-synaptic swelling that was beginning to obscure the endplate, and as severe if there was significant axonal and pre-synaptic swelling, which obscured the endplate (Chapter 2, Figure 2.3B). Quantification of the levels of pre-synaptic swelling was performed on four treatment groups: PACAP27 (182.49mg/kg) and saline treated *Smn*<sup>2B/-</sup> mice and PACAP27 (182.49mg/kg) and saline treated control littermates. Results showed that there was no significant difference in the number of endplates that showed no signs of pre-synaptic swelling between PACAP27 and saline treated *Smn*<sup>2B/-</sup> animals at disease end-stage (Figure 4.11C). There was also no significant difference between PACAP27 and saline treated *Smn*<sup>2B/-</sup> animals in the mild, moderate, or severe pre-synaptic swelling categories. As expected, there was a significant decrease in the endplates without pre-synaptic swelling between the saline treated *Smn*<sup>2B/-</sup> mice and their control littermates (Figure 4.11C). The same pattern could also be seen for the PACAP27 treated *Smn*<sup>2B/-</sup> mice and their respective control littermates. In both, the PACAP27 and saline treated, *Smn*<sup>2B/-</sup> mice, the majority of quantified endplates showed either mild or moderate pre-synaptic swelling. These findings suggest that the IP administration of purified PACAP27 protein has no beneficial effect on the NMJ pathology of the *Smn*<sup>2B/-</sup> mouse model. PACAP27

was not able to decrease overall NMJ pathology in the TVA muscle of the *Smn*<sup>2B/-</sup> mice, neither showing decreased levels of synaptic loss, nor causing an increase in endplates that are unaffected by pre-synaptic swelling in the *Smn*<sup>2B/-</sup> mice in comparison to their saline treated *Smn*<sup>2B/-</sup> control littermates.

Overall, findings show that daily treatment from P5 to end of life with 182.49mg/kg IP administered PACAP27 or 251.8mg/kg PACAP38 protein, has no significant effect on the *Smn*<sup>2B/-</sup> mouse model of SMA. Neither isoform of purified PACAP protein was able to improve the phenotype, increase PACAP levels in liver or brain, or to offer neuroprotection in the *Smn*<sup>2B/-</sup> animals. There was no significant improvement observed in the weight, motor performance, and NMJ pathology of the PACAP treated *Smn*<sup>2B/-</sup> animals in comparison to saline treated *Smn*<sup>2B/-</sup> mice. Since it is unclear if the half-life of the IP delivered purified PACAP protein was long enough to sustain elevated levels of PACAP over the entirety of the treatment period, or if there were fluctuations of the PACAP levels, further experiments with a different delivery route and system are needed before drawing final conclusions about the efficacy of PACAP as neuroprotective modifier in mouse models of SMA.

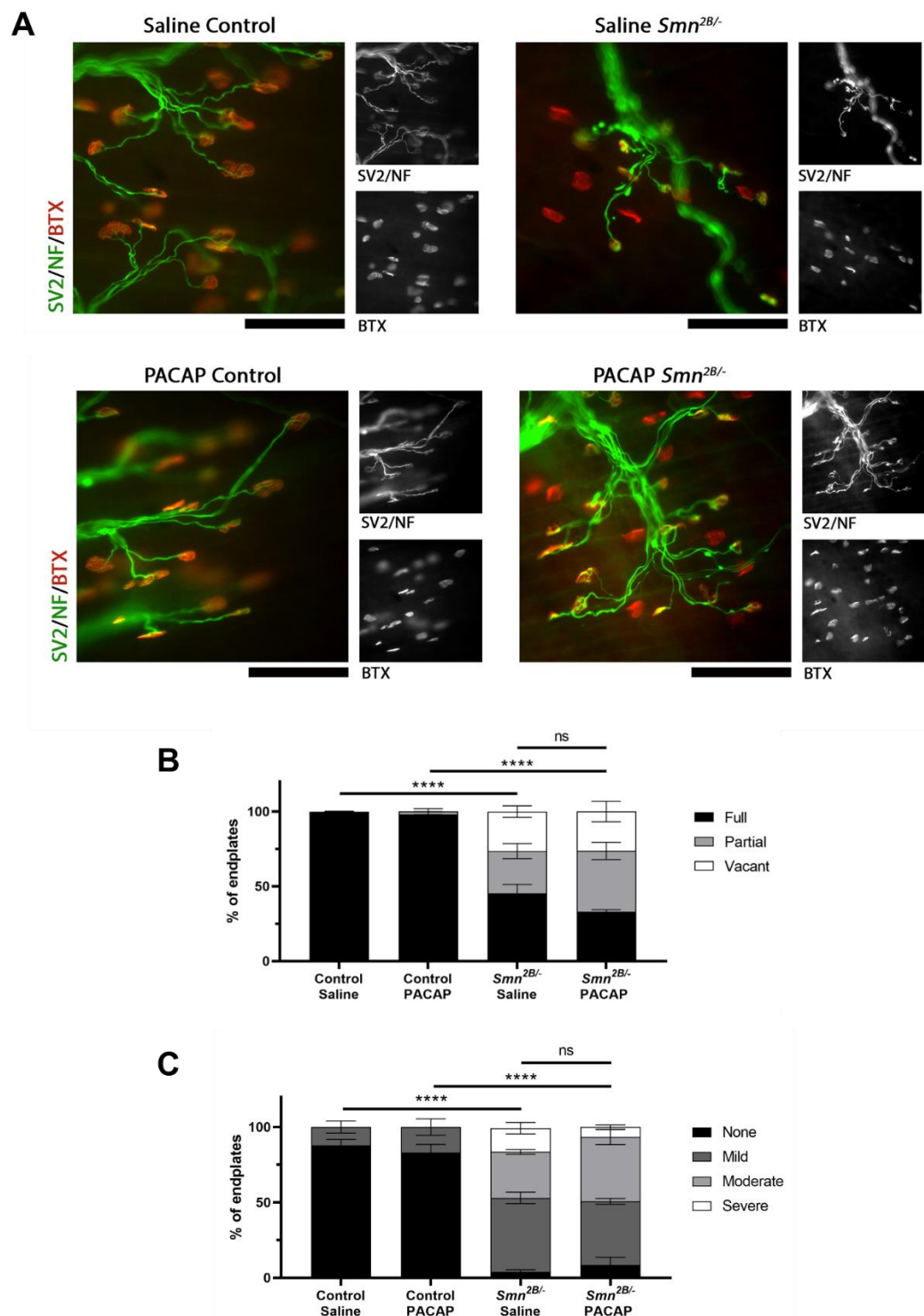


**Figure 4.11: PACAP27 treatment administered as daily intraperitoneal injections shows no decrease in NMJ pathology at disease end-stage in the *Smn*<sup>2B/-</sup> mouse model of SMA. (A)** Representative fluorescent microscope images showing NMJs with their pre-synaptic terminal labelled with antibodies against neurofilament (NF, 2H3; green) and synaptic vesicle protein 2 (SV2; green) and the motor endplates labelled with  $\alpha$ -Bungarotoxin (BTX; red) from transversus abdominis muscles (TVAs) in PACAP27 (182.49mg/kg) and saline treated *Smn*<sup>2B/-</sup> and control mice. Scale bar: 50 $\mu$ m. **(B)** Bar chart showing the quantification of percentage of full, partial and vacant endplates following daily PACAP27 or saline treatment in *Smn*<sup>2B/-</sup> mice compared to controls in the TVA muscle. Significant differences were only indicated for fully occupied endplates. Note that there is no significant difference in fully occupied endplates between PACAP27 and saline treated *Smn*<sup>2B/-</sup> mice. Two-way ANOVA

with Tukey correction (ns= no significance and \*\*\*\* $p \leq 0.0001$ ). n=2 for saline controls, n=4 for PACAP27 and saline *Smn*<sup>2B/-</sup> mice, and n=6 for PACAP27 controls. 3 FOV quantified directly under the microscope for one muscle each per n-number. Error bars represent mean  $\pm$  SEM. **(C)** Bar graph showing the quantification of percentage of levels of pre-synaptic swelling (severe, moderate, mild and none) following daily PACAP27 or saline treatment in *Smn*<sup>2B/-</sup> mice compared to controls in the TVA muscle. Significant differences were only indicated for endplates with no pre-synaptic swelling. Note that there is no significant difference in endplates with no pre-synaptic swelling between PACAP27 and saline treated *Smn*<sup>2B/-</sup> mice. Two-way ANOVA with Tukey correction (ns=no significance and \*\*\*\* $p \leq 0.0001$ ). n=2 for saline controls, n=4 for PACAP27 and saline *Smn*<sup>2B/-</sup> mice, and n=6 for PACAP27 controls. 3 FOV quantified directly under the microscope for one muscle each per n-number. Error bars represent mean  $\pm$  SEM.

#### **4.3.4. Investigating the neuroprotective properties of AAV9-PACAP treatment on the neuropathology of the *Smn*<sup>2B/-</sup> mouse model**

The treatment of animals from the *Smn*<sup>2B/-</sup> mouse model of SMA with intraperitoneally injected purified PACAP27 or PACAP38 protein, showed no significant improvement in disease phenotype or NMJ pathology in this model. A caveat of these experiments was that the half-life of the administered purified PACAP protein might have been too short, leading to high fluctuations in the daily PACAP levels of mice and failing to provide continuously elevated PACAP levels. To address this caveat, I next chose to utilise a different treatment delivery which has the potential to allow a more effective investigation of potential neuroprotective effects of PACAP in a mouse model of SMA. I therefore administered a single intracerebroventricular injection of a AAV9-PACAP viral vector to *Smn*<sup>2B/-</sup> mice and their control littermates, to guarantee a continuous elevation of PACAP levels in this mouse model of SMA until the end-stage of the disease. *Smn*<sup>2B/-</sup> mice and their control littermates received a single ICV injections with either a low dose ( $3.33 \times 10^{10}$  VG/mL) AAV9-PACAP, a high dose ( $8.07 \times 10^{12}$  VG/mL) AAV9-PACAP within the first three days of life or remained untreated. All animals were sacrificed at disease end-stage at P18. Animals were closely monitored and fluorescent intensity of PACAP and NMJ pathology analysis were performed on tissues collected from the different *Smn*<sup>2B/-</sup> mice treatment groups to quantify the effects of PACAP on neuropathology.

To verify if a single ICV injection with a low dose ( $3.33 \times 10^{10}$  VG/mL) or a high dose ( $8.07 \times 10^{12}$  VG/mL) of AAV-PACAP was able to cross the blood-brain

barrier and reach the central nervous system in the *Smn*<sup>2B/-</sup> mouse model of SMA, the fluorescent intensity of PACAP in motor neurons in the spinal cord was quantified. Fluorescent intensity levels of spinal motor neurons were obtained in collaboration with MSc student Christy Ip. Spinal cord motor neurons labelled with antibodies against PACAP27/38 were analysed in samples from control and *Smn*<sup>2B/-</sup> mice treated with either a low dose AAV9-PACAP, a high dose AAV9-PACAP or with saline (Figure 4.12). Results showed that there was no significant difference in the fluorescent intensity in motor neurons from PACAP treated control and *Smn*<sup>2B/-</sup> animals compared to their respective saline treated control groups when treated with a low dose of AAV9-PACAP (Figure 4.12A). Results further showed that there was no significant difference in the fluorescent intensity in motor neurons from PACAP treated control and *Smn*<sup>2B/-</sup> animals compared to their respective saline treated control groups when treated with a high dose of AAV9-PACAP (Figure 4.12B).

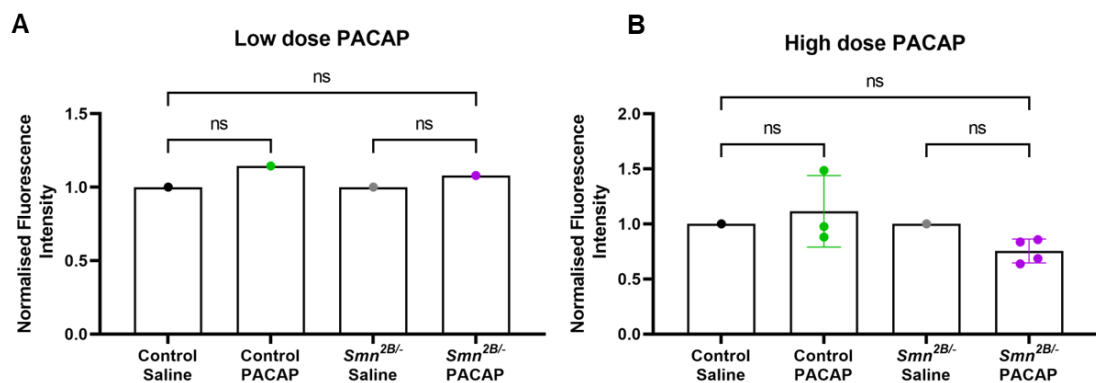


**Figure 4.12: AAV9-PACAP treatment administered as single ICV injection shows no significant increase in fluorescence intensity in spinal cord motor neurons of the *Smn*<sup>2B/-</sup> mouse model at disease end-stage.** Bar charts showing the quantification of normalised fluorescence intensity (**A**) in low dose AAV9-PACAP ( $3.33 \times 10^{10}$  VG/mL) and saline treated control and *Smn*<sup>2B/-</sup> samples and (**B**) high dose AAV9-PACAP ( $8.07 \times 10^{12}$  VG/mL) and saline treated control and *Smn*<sup>2B/-</sup> samples. All values are normalised to their respective saline treated control group. Note that there was no significant difference between saline and low or high dose AAV9-PACAP treated *Smn*<sup>2B/-</sup> mice. Kruskal-Wallis multiple comparisons test with Dunn's correction (ns = not significant). For low dose PACAP and saline treatment, an n-number of n=1 has been analysed for each genotype (9 images analysed per sample, 10 MNs analysed per image; 90 MNs in total). For high dose PACAP samples, each genotype a n number of n=3 for saline treated and PACAP treated controls, a n=1 for saline treated *Smn*<sup>2B/-</sup> and a n=4 for PACAP treated *Smn*<sup>2B/-</sup> samples has been analysed (3 images analysed per sample, 10 MNs analysed per image; 30 MNs in total). Error bars represent mean  $\pm$  SEM.

To determine the effect of a single ICV injection with a low dose ( $3.33 \times 10^{10}$  VG/mL) or a high dose ( $8.07 \times 10^{12}$  VG/mL) of AAV-PACAP on the neuropathology of the *Smn*<sup>2B/-</sup> mouse model of SMA, I quantified the level of NMJ loss that was present in the TVA muscle of treated and untreated *Smn*<sup>2B/-</sup> mice at disease end-stage. NMJ occupancy quantification was performed on three different groups: *Smn*<sup>2B/-</sup> mice treated with a low dose of AAV-PACAP, *Smn*<sup>2B/-</sup> mice treated with a high dose of AAV-PACAP, and untreated *Smn*<sup>2B/-</sup> mice (Figure 4.13). Results showed that there was no significant difference in the remaining fully innervated endplates between all three treatment groups of *Smn*<sup>2B/-</sup> animals, with 63.2% fully innervated endplates remaining in the untreated group, 56.3% fully innervated endplates remaining in the low dose AAV-PACAP group, and 60.1% endplates remaining in the high dose AAV-PACAP group (Figure 4.13B). There was also no significant difference in the number of vacant endplates at disease end-stage between any of the groups (Figure 4.13B). These findings suggest that the AAV9 administration of PACAP has no beneficial effect on the NMJ pathology of the *Smn*<sup>2B/-</sup> mouse model. Neither a low dose, nor a high dose of a single ICV AAV-PACAP treatment showed increased levels of PACAP in spinal motor neurons or was able to decrease neuropathology in the TVA muscle of the *Smn*<sup>2B/-</sup> mice in comparison to their untreated control littermates.

Overall, findings show that a single ICV AAV9-PACAP treatment with up to  $4 \times 10^{10}$  GC/animal, seems to have no significant effect on the neuropathology of the *Smn*<sup>2B/-</sup> mouse model of SMA.

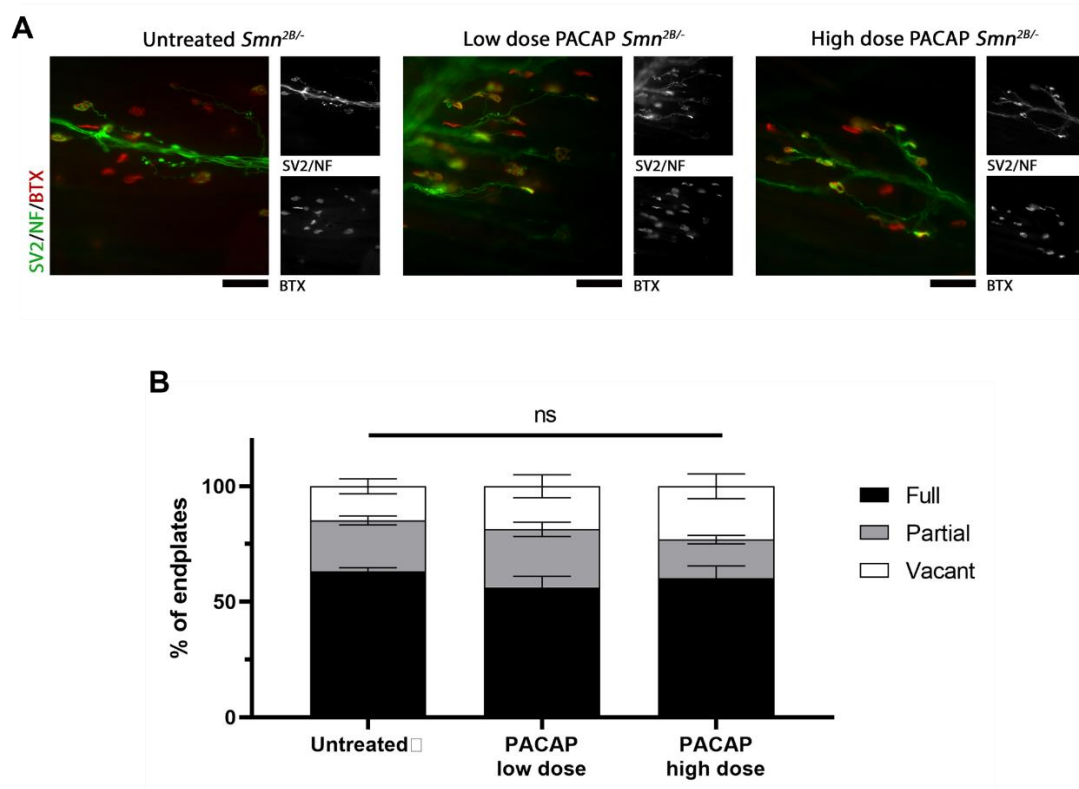


**Figure 4.13: AAV9-PACAP treatment administered as single ICV injections shows no significant difference in synaptic loss in the *Smn*<sup>2B/-</sup> mouse model of SMA. (A)** Representative fluorescent microscope images showing NMJs with their pre-synaptic terminal labelled with antibodies against neurofilament (NF, 2H3; green) and synaptic vesicle protein 2 (SV2; green) and the motor endplates labelled with  $\alpha$ -Bungarotoxin (BTX; red) from transversus abdominis muscles (TVAs) in untreated *Smn*<sup>2B/-</sup> animals, in low dose PACAP treated *Smn*<sup>2B/-</sup> animals, and in high dose PACAP treated *Smn*<sup>2B/-</sup> animals. Scale bar: 50 $\mu$ m. **(B)** Bar graph showing the quantification of percentage of full, partial and vacant endplates in the TVA muscle in the *Smn*<sup>2B/-</sup> mouse model. Mice were either untreated, treated with a low dose of AAV9-PACAP (3.33 $\times 10^{10}$ VG/mL), or treated with a high dose of AAV9-PACAP (8.07 $\times 10^{12}$ VG/mL). Significant differences were only indicated for fully occupied endplates. Note that there is no significant difference in the number of fully occupied endplates between any of the three groups. Two-way ANOVA with Tukey correction (ns= no significance and \*\*\*\* $p \leq 0.0001$ ).  $n=4$  for untreated *Smn*<sup>2B/-</sup> mice,  $n=5$  for low dose treated *Smn*<sup>2B/-</sup> mice, and  $n=4$  for high dose treated *Smn*<sup>2B/-</sup> mice. 3 FOV quantified directly under the microscope for one muscle each per  $n$ -number. Error bars represent mean  $\pm$  SEM.

#### 4.3.5. Skeletal Muscle Publication Declaration

Some of the work presented within this chapter of this thesis has been published as a research paper in *Skeletal Muscle*. The publication is entitled: Mouse models of SMA show divergent patterns of neuronal vulnerability and resilience (Woschitz et al., 2022). A copy of this publication is provided as Appendix 1 of this thesis.

## 4.4. Discussion

### 4.4.1. Overview of results

In this chapter of this thesis, I performed two distinct cross-model transcriptional comparisons of differentially vulnerable motor neurons across mouse models of SMA to identify refined lists of potential neuroprotective modifiers in SMA. I firstly conducted a cross-model transcriptional analysis on differentially vulnerable motor neurons from the CN VII from the SMN $\Delta$ 7 and the *Smn*<sup>2B/-</sup> mouse model of SMA at a pre-symptomatic time point. By comparing transcriptional profiles from healthy mice and animals with a SMA phenotype from both mouse models, I showed that an increase in transcripts associated with ribosomal bioenergetics, vesicle trafficking and neuronal development is correlative with motor neuron protection. Secondly, I conducted a cross-model transcriptional analysis on differentially vulnerable motor neurons from neurologically normal animals of the SMN $\Delta$ 7 and the *Smn*<sup>2B/-</sup> mouse model of SMA. I identified a refined list of potential neuroprotective modifiers, including PACAP. I tested the effects of PACAP on weight, phenotype, and neuropathology *in vivo* in the *Smn*<sup>2B/-</sup> mouse model of SMA. My results showed that neither a single intracerebroventricular delivery of AAV9-PACAP nor a daily intraperitoneal delivery of purified PACAP protein had a significant effect on phenotype and neuropathology in a mouse model of SMA. Overall, in this chapter I provided a list of candidate modifiers and presented two possible workflows to test neuroprotective effects on phenotype and neuropathology of modifiers of interest *in vivo*.

### 4.4.2. Identified molecular pathways and their potential for motor neuron protection

By performing a cross-model transcriptional analysis of differentially vulnerable CN VII motor neurons in healthy and SMA mice, I demonstrated that certain groups of transcripts are candidate modifiers of motor neuron protection. The conducted analysis revealed an enrichment in transcripts associated with the Gene Ontology (GO) term 'ribonucleoprotein complex biogenesis'. Transcripts in this GO term cluster contribute to regulation of the translation apparatus, ribosome biogenesis as part of the ribosomal proteins from the small and large subunit, as well as to pre-RNA and rRNA processing and maturation. Previous

studies have shown that SMN and associated proteins are implicated in pre-mRNA splicing as well as in the regulation of translation (Lauria et al., 2020; Martinez-Salas et al., 2020; Sanchez et al., 2013; Singh et al., 2017). A decrease or loss of SMN induces depletion of the ribosome, thereby impairing proteins that are associated with motor neuron stability and function (Lauria et al., 2020). It can therefore be hypothesised that a change in regulation of these ribosomal pathways in resistant motor neurons could render them less vulnerable to reduced SMN levels.

The conducted cross-model transcriptional analysis further showed an enrichment in the transcripts associated with the GO term 'protein localisation to cell periphery'. Transcripts in this cluster are associated with cellular pathways concerned with vesicle trafficking, microtubule dynamics and neurite outgrowth. A number of transcripts from this cluster, including *Rap1a*, *Prkci*, *Magi2*, *Hras*, and *Numa1*, are part of the *Ras* and *Rap1* signalling pathways. The *Ras* signalling pathway transduces signals from the extracellular milieu to the cell nucleus, activating specific genes for cell growth, division and differentiation. Ras-associated protein 1 (*Rap1*; small G protein belonging to the Ras superfamily) and its associated *Rap1* signalling pathway, control many important cellular processes, such as cell junction, cell migration, cell proliferation and cell survival. The transcripts *Magi2*, *Kif5b*, *Sqstm1* and *Rap1a* are associated with neuronal development, including neurite outgrowth, axonal transport and elongation, and microtubule motor activity. Some of these transcripts have previously been implicated in the pathogenesis of neurodegenerative diseases. *Kif5b* is a member of the kinesin motor family and is involved in anterograde axonal transport. As mutations in the axonal transport motor proteins can lead to neurological disorders, signalling pathways associated with the kinesin motor family have been implicated in various neurodegenerative diseases including Huntington's disease, Parkinson's disease and ALS. Another member of the kinesin motor family, *Kif5a*, has recently been identified as an ALS-associated gene with genome-wide significance (Nicolas et al., 2018). It has been shown to contribute to the disruption of axonal transport in the pathogenesis of ALS (Nicolas et al., 2018). It has further been demonstrated that gene mutations associated with an ALS

mouse model interfere with kinesin members, thereby facilitating defects in axonal transport that ultimately lead to motor neuron death (Shi et al., 2010). The association of the kinesin family members with axonal transport defects and motor neuron degeneration, suggests that *Kif5b* and other kinesin members have the potential to also contribute to the pathogenesis of other motor neuron diseases like SMA. Furthermore, as mentioned above, several of the identified transcripts have an important role in axon function. Since SMA is an axonopathy where SMN has pivotal functions in the axon health and pathology, several of the above-mentioned genes show the potential to be regulated as compensation for the loss of SMN to maintain axonal function (Dombert et al., 2014; Fallini et al., 2012, 2011; Pagliardini et al., 2000; Pletto et al., 2018). Further, some of the identified transcripts in this screen are associated with neurite outgrowth. SMN is described as a RNA binding protein that traffics mRNAs to the distal axons and synapses, contributing to the localisation of one of its cargos,  $\beta$ -actin, to growth cones (Rossoll et al., 2003). It was shown that SMN deficiency led to impairments in neurite outgrowth and the localisation of  $\beta$ -actin mRNA to growth cones (Rossoll et al., 2003), thereby confirming that actin dynamics play an important role in growth cone formation and neurite outgrowth. In conjunction with this, some of the above-mentioned transcripts could contribute to the regulation of neurite outgrowth defects in SMA.

Another transcript that was enriched in the 'protein localisation to cell periphery' cluster of this transcriptional analysis is *Sqstm1*. *Sqstm1* is associated with mitophagy, the removal of damaged mitochondria through the autophagy processes. The *Sqstm1* gene encodes the p62 protein, a major pathologic protein involved in neurodegeneration. Mutations in the *Sqstm1* gene have previously been reported in both ALS and the related disorder frontotemporal dementia. Previous work showed that a p62 deficiency was associated with an inhibition in complex I mitochondrial respiration, which was due to deficits in the electron transport chain (Bartolome et al., 2017). A silencing or knockout of p62 in animal models of ALS resulted in mitochondrial dysfunction (de Castro et al., 2013; Lee and Shin, 2011; Seibenhener et al., 2013). These findings taken together show that dysfunction of the *Sqstm1*

gene demonstrate a strong association with pathophysiological events associated with energy metabolism. Further, mitochondrial bioenergetics status was previously found to be implicated in motor neuron vulnerability (Boyd et al., 2017; Kline et al., 2019b). I therefore suggest that the upregulation of *Sqstm1* could function as a protective modifier of motor neuron pathology in SMA. Increasing the expression of *Sqstm1* and other potential modifiers that were identified in this analysis, will elucidate their potential neuroprotective properties in *in vivo* models of SMA.

#### **4.4.3. Identified potential candidate modifiers for neuroprotection**

By conducting a cross-model transcriptional analysis of various wildtype motor neuron populations, which are differentially vulnerable across SMA mouse models, I identified a refined list of modifiers that might confer motor neuron protection in SMA. The performed analysis revealed a list of 25 significantly altered transcripts that show a common directional change across mouse models of SMA. Various identified genes are associated with functional clusters such as synapse, extracellular matrix, and synaptic vesicle membrane. Upon closer observation of the refined list of potential neuroprotective modifiers, a number of transcripts demonstrate the potential to be candidate modifiers of motor neuron protection. These candidate genes include *Col5a2*, *Gabra1*, *Gira1*, and *Adcyap1*. The *Col5a2* gene encodes one of the low abundance fibrillar collagens and the gene is associated with cellular functions of the extracellular matrix. Mutations in other collagens, like *Col4a1* and *Col4a2*, can cause neuromuscular dysfunction, myopathies and neurological disorders. Collagens represent a large component of the extracellular matrix, which is important for signal transduction and the functional and structural integrity of NMJs (Cescon et al., 2018). Previous work showed that another collagen (*ColVI*) is present within the synaptic cleft of NMJs, maintaining the postsynaptic apparatus (Cescon et al., 2018). They further demonstrate that a deficiency of *ColVI* results in an impairment in NMJ function and structure in their *in vivo ColVI* knockout mouse model (Cescon et al., 2018). These findings promote the idea that other collagens, such as *Col5a2*, could also alter NMJ function and have an involvement in NMJ

maintenance, thereby demonstrating an interesting candidate modifier for further investigation.

The cross-model transcriptional analysis further identified *Gabra1* as candidate modifier of motor neuron protection. *Gabra1* is associated with the functional cluster 'synapse'. The gene encodes a receptor for gamma-aminobutyric acid (GABA), which is the major inhibitory neurotransmitter in the mammalian brain. Previous work has shown that dysregulation of *Gabra1* and GABA receptors has implications on the neuropathology of selective vulnerability of motor neurons in ALS (Brockington et al., 2013; Comley et al., 2015; Lorenzo et al., 2006). It has been shown that ALS mice present with an insufficient synaptic inhibition, modulated through glycine receptors (GlyR) and GABA<sub>A</sub> receptor expression (Lorenzo et al., 2006). Work on control animals and control patients in ALS showed evidence that reduced GABA-mediated synaptic transmission is a driver of selective vulnerability in spinal motor neurons (Brockington et al., 2013). Further, a study indicated *Gabra1* as candidate for oculomotor resistance in ALS mice and patients (Comley et al., 2015). High levels of *Gabra1* were found in oculomotor motor neurons in end-stage ALS patients, in contrast to low *Gabra1* levels in vulnerable motor neurons of other brain regions. The above-described findings implicate *Gabra1* in the protection of motor neurons in ALS, thereby suggesting this as a promising candidate modifier for motor neuron protection in SMA.

Furthermore, *Gla1* was identified as modifier candidate in the transcriptional analysis in this chapter. The protein encoded by *Gla1* is part of the  $\alpha 1$  subunit of a pentameric inhibitory glycine receptor (GlyR). GlyR  $\alpha 1$  mediates postsynaptic inhibition in the mammalian CNS and defects in this GlyR subunit have been associated with various neurological disorders. Previous work demonstrated that a knockout of the  $\alpha 1$  subunit of GlyR resulted in a severe motor phenotype in zebrafish (Samarut et al., 2019). While other alpha subunits did not lead to developmental deficits in motor function, the importance of *Gla1* for correct glycine inhibitory neurotransmission controlling locomotion was demonstrated (Samarut et al., 2019). It was further shown that GlyR trafficking occurs, amongst others, via binding to the motor proteins

kinesin superfamily protein 5 (KIF5) (Maas et al., 2009). This is an interesting link, since, as discussed earlier, *Kif5a* is an ALS-associated gene of the kinesin family that facilitates defects in axonal transport, ultimately leading to motor neuron death (Nicolas et al., 2018; Shi et al., 2010). Coupled with the above-described findings that GlyR expression modulates an insufficient synaptic inhibition in ALS mice (Lorenzo et al., 2006), it can be suggested that *Glra1* has the potential to function as a neuroprotective modifier in SMA.

Lastly, the cross-model transcriptional analysis identified *Adcyap1* as candidate modifier of motor neuron protection. *Adcyap1* encodes a neuropeptide called pituitary adenylate cyclase-activating peptide (PACAP) and it is associated with a functional enrichment for synaptic vesicle membrane. PACAP belongs to the vasoactive intestinal polypeptide (VIP)-secretin-glucagon peptide superfamily (Arimura and Shioda, 1995). PACAP binds to the PAC1 receptor which inherits an important function in the central and peripheral nervous system (PNS), and it additionally binds to the two VIP receptors VPAC1 and VPAC2. There are two transcript variants of PACAP, PACAP27 and PACAP38, which are named according to their number of amino acids at the N-terminus (Arimura, 1992). PACAP inherits the function of a master regulator of the stress response, thereby maintaining homeostasis in the CNS and PNS. Functions of PACAP are associated with neuronal development, neuronal proliferation and differentiation, and neuroprotection (Rivnyak et al., 2018). PACAP was shown to have neuroprotective properties in ischemic stroke rodent models when administered via intracerebroventricular injection, intravenous injection or intraperitoneal injection (Fang et al., 2020). It was further demonstrated that PACAP offers potent neuroprotection in various neurodegenerative disease models, including Huntington's disease (Cabezas-Llobet et al., 2018), Parkinson's disease (Reglődi et al., 2004), spinal cord injury (Tsuchida et al., 2014), and spinobulbar muscular atrophy (Polanco et al., 2016). Neuroprotective effects of PACAP could furthermore be shown in various *in vivo* and *in vitro* models of ALS (Bonaventura et al., 2018; D'Amico et al., 2020; Maugeri et al., 2019; Ringer et al., 2013). In the SOD1 mouse model of ALS, PACAP was shown to protect against pre-ganglionic visceromotor neuron loss (Ringer et al., 2013).

PACAP was also demonstrated to rescue motor neuron degeneration following growth factor deprivation with a significant rescue effect on apoptotic cells in iPSCs of ALS (Bonaventura et al., 2018). Further, PACAP was shown to increase viability of cells, decrease apoptosis and to have a positive effect on the autophagy process of cells in a motor neuron-like model of ALS (D'Amico et al., 2020; Maugeri et al., 2019). Research on the effects of PACAP on axon regeneration, demonstrated that a PACAP knockout in mice led to a decrease in regenerating axon number after injury (Zhou et al., 1999). Further, an increase of the mRNA of PACAP was shown in motor neurons after injury, demonstrating that PACAP is involved in the injury response of motor neurons (Pettersson et al., 2004). Previous work also demonstrated that a deficiency in PACAP leads to a significant delay of the regeneration process of axons (Armstrong et al., 2008). These findings, coupled with the promising findings of neuroprotective properties of PACAP throughout various neurodegenerative disorders including ALS, suggest that PACAP is an interesting candidate modifier for *in vivo* investigations in SMA.

#### **4.4.4. PACAP as potential neuroprotective modifier in SMA**

I administered purified PACAP27 or PACAP38 protein via daily intraperitoneal (IP) injections (from P5 until end of life) to *Smn*<sup>2B/-</sup> mice and their control littermates, to investigate the potential effects on weight, motor phenotype and neuropathology. The *Smn*<sup>2B/-</sup> mouse model of SMA was chosen for these experiments because of its unique qualities in comparison to severe SMA mouse models. The *Smn*<sup>2B/-</sup> mouse model (Bowerman et al., 2012a; Hammond et al., 2010) is an intermediate model of SMA that has a prolonged pre-symptomatic period, as NMJ and motor neuron pathology start around P10. This model therefore allowed a daily treatment with PACAP for several days before onset of the neuropathology, while additionally offering the option of treatment and monitoring throughout a symptomatic period of up to ten days. Results showed that daily IP injections of PACAP27 or PACAP38 did not have any protective effects on motor phenotype or levels of synaptic loss in this mouse model. Results further showed that IP administration of PACAP27 and PACAP38 did not increase fluorescent intensity in liver samples. However, a non-significant trend towards an increase of fluorescent intensity was shown

in the brain samples of the PACAP27 treated animals compared to the saline treated animals, suggesting that the PACAP27 isoform might have crossed the blood-brain barrier and possibly entered the cells. One of the reasons why the administered PACAP treatment did not show any effect *in vivo*, could be the half-life of the injected PACAP protein. A study showed that PACAP38 was rapidly metabolised with a half-life of less than 5 minutes *in vitro* (Bourgault et al., 2008). However, the same study also showed that PACAP27 remained stable with no signs of degradation after 45 minutes *in vitro* (Bourgault et al., 2008). This could indicate that the half-life of PACAP could be too short, causing a high fluctuation of PACAP levels throughout the day and a not stable enough increase in PACAP levels to exert neuroprotection *in vivo*. Interestingly, other studies have seen protective effects of PACAP in other research areas when administered intraperitoneally once or on a daily basis (Lin et al., 2015; Vu et al., 2015; Yada et al., 2000). Effective administration routes of PACAP in stroke also include intravenous, intracerebroventricular and intraperitoneal injections (Fang et al., 2020). Another reason for the lack of improvement of phenotype and neuropathology following PACAP treatment, could be the concentration of the administered PACAP protein. Interestingly, a review demonstrated that the dosage of single and continuously injected PACAP varies greatly between studies in stroke, already facilitating neuroprotection in doses as little as 1pM/mouse (Fang et al., 2020). In an effort to induce neuroprotection of PACAP in our *in vivo* model of SMA, a slightly higher dosage (100nM/kg) than the administered dosages in the above-mentioned review was chosen for this body of work. This 100nM/kg dosage was also utilised in previously published *in vitro* work on iPSC-derived motor neurons in a model of ALS (Bonaventura et al., 2018).

While exogenous administration of purified PACAP protein is non-invasive and cost effective and therefore a tempting delivery method for *in vivo* testing of a potential modifier, other delivery systems and routes may prove more effective in this mouse model of SMA. To further investigate the potential neuroprotective effects of PACAP on the neuropathology of the *Smn*<sup>2B/-</sup> mouse model of SMA, I chose an alternative delivery system and delivery route for the treatment. *Smn*<sup>2B/-</sup> mice and their control littermates were treated with a

single intracerebroventricular (ICV) injection with either a low dose ( $3.33 \times 10^{10}$  VG/mL) or a high dose ( $8.07 \times 10^{12}$  VG/mL) of PACAP administered via a GFP tagged viral AAV9 vector. Adeno-associated viruses (AAV) are small (25nm) viruses that belong to the Parvoviridae family. AAV viruses can package a linear single-stranded DNA genome and deliver the stored genetic information to post-mitotic cells, like neurons (Daya and Berns, 2008). There are 13 different serotypes of AAV viruses (AAV1-AAV13), each displaying variations in tropism and efficacy of transduction in various tissues (Kantor et al., 2014). AAV9 viral vectors were found to display the greatest tropism in the central nervous system (Kantor et al., 2014). A previous study further demonstrated that the use of ICV injections of a AAV9 gene therapy in SMA was superior to intravenous injections, thereby showing improvements in NMJ and motor function (Shababi et al., 2018). These characteristics make the AAV9 viral vector delivered via an ICV injection an attractive delivery system for gene therapies, and here the delivery system of choice for the investigation of PACAP as neuroprotective modifier. This delivery route was especially attractive in this context, as it promised a direct delivery of PACAP to the CNS, in addition to providing an opportunity to elevate PACAP levels in these animals and sustain continuous elevation until disease end-stage. My results showed that the ICV administration, of both a low and a high concentration, of AAV9-PACAP did not increase the fluorescent intensity of PACAP observed in spinal cord motor neurons and had no significant effects on the neuropathology in the *Smn*<sup>2B/-</sup> mouse model. AAV9 viral vectors were effectively utilised for the testing of several already approved SMA treatments, as well as for a number of neuroprotective modifiers in mouse models of SMA (Bogdanik et al., 2015; Comley et al., 2022; Glascock et al., 2012; Hammond et al., 2016; Le et al., 2011; Lutz et al., 2011; Robbins et al., 2014). The effects of SNCA (Kline et al., 2017), STMN1 (Villalón et al., 2019), and SYT13 (Nizzardo et al., 2020) were tested via AAV9 delivery, all showing significant improvements in survival, weight, and neuropathology in mouse models of SMA. It therefore seems unlikely that the delivery system is responsible for the inability to observe a treatment effect from PACAP. A more likely reason for the failure of PACAP to exert neuroprotective properties, is the concentrations used for the treatment in these experiments. Previous studies investigating

neuroprotective modifiers utilised a concentration of  $1 \times 10^{11}$ VG/animal (Villalón et al., 2019) in a SMA mouse model, and one injection of  $1.25 \times 10^{11}$ VG/animal or  $2.5 \times 10^{11}$ VG/animal as low dose, or two injections as a higher dose in a model of spinal muscular atrophy with respiratory distress type 1 (Shababi et al., 2016). Here, due to the titre of the purchased viral vector, injection of PACAP could only be performed with either a low dose of  $1 \times 10^8$ VG/animal, or a high dose of  $4 \times 10^{10}$ VG/animal. These concentrations may have been too low to exert the neuroprotective effect of PACAP in this mouse model of SMA. Additionally, the possibility remains that the chosen parameters for investigation of the effect of PACAP on the neuropathology of a SMA mouse model, were not ideal. Not choosing the best possible parameters for investigation, increases the likelihood of missing more subtle changes in phenotype and neuropathology. However, considering that the AAV9-PACAP fluorescent intensity quantification in spinal cords did not show any significant differences between PACAP treated and saline treated animals with either the low or the high dose, it is possible that the virus was not able to reach the motor neurons and unfold its therapeutic potential. A few possibilities remain as to why the PACAP treatment was not working, one of them being that the injected virus was faulty and not working, and that therefore no increase in PACAP levels could be achieved in the CNS. As discussed above, other possibility could be that the chosen dosage was too low to increase PACAP levels. I suggest that further steps are taken to perform a comprehensive analysis of the potential neuroprotective effects of PACAP in SMA, that could not yet be completed within the timeframe of this PhD project. To determine if PACAP levels were indeed increased in AAV9-PACAP treated animals but were not able to be detected by measuring the PACAP fluorescent intensity in motor neuron of the spinal cord, western blot analysis of the relative protein levels of PACAP in brain and spinal cord should be performed. Evidence of changes in relative PACAP protein levels can inform further about the actual ability of the treatment to deliver PACAP to the CNS. Furthermore, a comprehensive analysis of motor performance in treated and untreated animals should be conducted. Several motor tests and motor milestones should be recorded throughout the lifespan of the animals to detect subtle changes in motor phenotype. Further work detailing the effect of PACAP on

phenotype, protein expression levels of PACAP, and other aspects of neuropathology is required to draw final conclusions about the efficacy of PACAP as neuroprotective modifier in mouse models of SMA. However, the possibility that PACAP is in fact not a neuroprotective modifier in SMA persists. Even though PACAP was shown to have strong neuroprotective properties in ALS and thereby in a related motor neuron disease, the underlying disease mechanisms of both disorders differ greatly. While the disease mechanism in ALS is closely linked to the elevation of inflammatory markers, neuroinflammation is not part of the disease mechanism in SMA. Modifiers that are effective in one motor neuron disease or neurodegenerative disease might not have neuroprotective properties in closely related diseases due to their differences in underlying disease mechanisms.

#### **4.4.5. Conclusions**

Overall, the work in this chapter demonstrates that cross-model transcriptional comparisons, in both neurologically normal mice and animals with disease phenotype, facilitate the identification of refined lists of potential neuroprotective modifiers in mouse models of SMA. Further work is required to conduct comprehensive testing *in vivo* and *in vitro* of more identified modifiers that could confer motor neuron protection to determine their actual therapeutic potential in SMA.

## Chapter 5

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### **Optimisation of single cell LCM-sequencing to identify cellular signature profiles of facial nucleus motor neurons in a mouse model of SMA**

#### **5.1. Chapter overview and key findings**

In this thesis, I identified significant divergence in relative vulnerability in the neurons of the facial motor nucleus (CN VII). In this chapter, I aim to utilise this divergence to perform high spatial resolution transcriptional profiling of motor neurons in the SMN $\Delta$ 7 mouse model. The Hedlund laboratory has pioneered the LCM-seq method, which combines laser capture microdissection (LCM) for precise isolation of cells from post-mortem tissue, with polyA-based Smart-seq2 RNA-sequencing (Nichterwitz et al., 2018, 2016). Here, I optimised and applied this LCM-seq method to perform RNA-sequencing on single motor neurons isolated from the CN VII of the brainstem from the SMN $\Delta$ 7 mouse model. Around 60 single motor neurons have been isolated from each CN VII nucleus from pre-symptomatic (P2), early symptomatic (P5) and late-symptomatic (P9) disease time points of SMN $\Delta$ 7 mice and respective controls. cDNA library preparation protocols have been optimised to produce 1049 cDNA libraries from collected motor neurons. Lastly, a subset of 24 single cell libraries has been barcoded and sent for MiSeq sequencing. Results showed that I was able to produce good quality cDNA libraries with the optimised single cell LCM-seq protocol that passed sequencing quality control.

The principal findings of the following chapter are:

- Reliable identification of the CN VII in brainstems of mice was achieved, enabling single motor neuron isolation with LCM.
- Optimisation of the single cell LCM-seq method allowed cDNA library preparation for sequencing with a success rate of 83%.
- MiSeq sequencing of a subset of prepared samples confirmed production of good quality single cell libraries for further in-depth sequencing of collected motor neurons.

## 5.2. Introduction

Transcriptomic analysis like RNA-sequencing is a popular method to investigate the unique transcriptome of various cell types. Utilising RNA-sequencing methods facilitates the study of a variety of cellular aspects, including cell specific mechanisms in health and disease, and the identification of altered transcripts and molecular pathways. While conventional bulk RNA-seq is only able to produce an average expression signal for an entirety of sequenced cells, single cell RNA-sequencing (scRNA-seq) enables the more precise analysis and understanding of the transcriptome from individual cells. Through single cell RNA-sequencing, it has been demonstrated that seemingly homogenous cell populations, in fact showed a large heterogeneity (Shalek et al., 2014; Wilkinson, 2009). This opportunity for a more precise understanding of the subpopulations within specific cell types and the individual transcriptomes of single cells is essential for understanding their unique roles in cellular function. For this reason, single cell RNA-seq has become a rapidly expanding field since the first publication of a single cell transcriptome analysis in 2009 (Tang et al., 2009).

In the past decade a variety of different high and low throughput scRNA-seq protocols have been developed to look at the transcriptomes from very small amounts of biological tissue (Hashimshony et al., 2012; Islam et al., 2012, 2011; Picelli et al., 2013; Sasagawa et al., 2013; Tang et al., 2010). High throughput methods include Drop-Seq, 10xChromium, and Seq-well, while popular low throughput methods are Smart-seq2 and CEL-seq2 (see Ding et al., 2020 for comprehensive review of scRNA-seq methods). While popular scRNA-seq methods rely on profiling of 3' ends of RNA or template switching, Smart-seq2 offers full-length transcriptome profiling of poly(A)<sup>+</sup> RNA from single cells (Picelli et al., 2014, 2013). With Smart-seq2, Picelli et al. have optimised the Smart-Seq method to improve sensitivity, accuracy, and full-length coverage across single cell transcripts. This method is cost effective through the use of off the shelf reagents and offers improved detection, coverage and accuracy, increasing the sensitivity to gene expression detection and decreasing the variability in single cell transcriptome data from different cell types and amounts (Picelli et al., 2013). While Picelli et al. utilises Smart-

Seq2 by isolating cells disaggregated into a single cell suspension or manual cell picking with a micro capillary pipette (Picelli et al., 2014), Schweingruber et al. utilises fluorescence-activated cell sorting (FACS) before following the Smart-Seq2 protocol for library preparations (Schweingruber et al., 2023). This shows that differences in various scRNA-seq protocols do not just arise from their throughput amount or transcript coverage length, but also from their single cell isolation method. Other popular isolation methods for single cell sequencing are limiting dilution, micromanipulation, microfluidic technology and laser capture microdissection (as reviewed by Hwang et al., 2018).

In 2016, Nichterwitz et al. published a newly developed method for precise spatial transcriptomic profiling (Nichterwitz et al., 2016). This method coupled laser capture microdissection (LCM) with a full-length mRNA-sequencing protocol and was termed LCM-seq. Even though others have previously utilised LCM as isolation method coupled with RNA extraction methods to obtain transcriptomic analysis from microarrays or bulk RNA-sequencing (Brockington et al., 2013; Hedlund et al., 2010; Kaplan et al., 2014; Murray et al., 2015), the LCM-seq method introduced a novel combination of isolation method and utilised library preparation protocol (Nichterwitz et al., 2016, 2018). Nichterwitz et al. coupled LCM with the poly(A)<sup>+</sup> based Smart-seq2 protocol (Picelli et al., 2014, 2013) that was developed for full-length RNA-sequencing for single cell transcriptomics. They further optimised several aspects to create a protocol where a small number of isolated cells per sample (between 1-120 cells) is sufficient to achieve good sequencing results. Lastly, they developed a direct lysis method that removed the need to conduct an RNA extraction from collected cells and therefore lowered technical noise in sequencing. In their method description paper, Nichterwitz et al. isolated and sequenced motor neuron cell pools comprised of 2-120 cells, as well as 13 single motor neurons from frozen mouse tissue (Nichterwitz et al., 2016). They demonstrated a 62% success rate to prepare cDNA libraries from their 13 isolated and sequenced single motor neurons. They further demonstrated that in both, single large spinal motor neurons (500-750 $\mu\text{m}^2$ ) and single small motor neurons from cranial nerve nuclei (130-300 $\mu\text{m}^2$ ), less genes were detected than when sequencing pooled samples. However, the number of detected

genes was comparable with live single spinal motor neurons derived from mouse embryonic stem cells. The described LCM-seq method was previously utilised for bulk collection of motor neurons from the brain and spinal cord, identifying import cellular pathways and modifier transcripts in the SMN $\Delta$ 7 mouse model of SMA (Nichterwitz et al., 2020). Even though Nichterwitz et al. demonstrate that their protocol cannot only be utilised for bulk RNA-sequencing, but that it can also be utilised for single cell RNA-sequencing (Nichterwitz et al., 2016), it is not yet routinely being used for scRNA-seq.

In chapter 3 of this thesis, I investigated and compared the selective vulnerability pattern present in different body regions of mice from four different SMA mouse model. My results showed that the head muscles in the SMN $\Delta$ 7 mouse model of SMA displayed a high heterogeneity of selective vulnerability within the same body region (see Chapter 3, Figure 3.9). Findings from other groups and my own results showed that the AAL, AS, LALc and masseter muscles are vulnerable, while the LALr and digastric posterior are comparatively resistant in this mouse model (Ling et al., 2012; Murray et al., 2008). This selective vulnerability within the same body region is particularly interesting, as all of these investigated muscles are innervated by the CN VII (facial) motor nucleus. Previous studies have utilised bulk sequencing of the CN VII in different mouse models of SMA to investigate transcriptional changes between differentially vulnerable motor neuron populations (Murray et al., 2015; Nichterwitz et al., 2020). While bulk RNA-sequencing is a useful tool to compare the transcriptomes from different cell populations, it has its limitations when trying to decipher the transcriptional differences within a highly heterogeneous cell population. Bulk RNA-sequencing can only outline the collective mean expression levels of the entirety of all cell types that are represented within the CN VII, while a single cell RNA-sequencing approach can be utilised as a more targeted approach to investigate the transcriptional differences between subpopulations. The selective vulnerability of the CN VII led me to hypothesise that the motor neuron population within the CN VII of the SMN $\Delta$ 7 mouse model, shows a high heterogeneity in motor neuron subpopulations. Some of these subpopulations appear to be resistant to degeneration, while others appear to be highly vulnerable during disease

progression. These differences in selective vulnerability constitute the basis to investigate if certain motor neuron subpopulations consist of specific cellular signatures that contribute to motor neuron protection in SMA.

In this chapter of this thesis, I aimed to optimise the existing LCM-seq method to be used as robust protocol for the use with LCM isolated single cell transcriptomic analysis. The expansion and optimisation of this method will allow the investigation of cellular signature profiles from CN VII motor neurons in a mouse model of SMA. Specifically, I aimed to look at the cellular signature profiles of motor neuron subpopulations that have the potential to contribute to motor neuron protection in a SMA mouse model. Identifying neuroprotective transcripts could eventually aid the identification of new targets for therapeutic strategies. In this body of work, I was able to find an approach that allowed the reliable identification of the CN VII in the brainstems of mice from the SMN $\Delta$ 7 mouse model. This enabled the isolation of single motor neurons utilising LCM. Further, I showed that the optimisation of the single cell LCM-seq method allowed the effective preparation of cDNA libraries for sequencing. Lastly, a subset of prepared cDNA libraries was sequenced on the MiSeq sequencing platform. This confirmed the production of good quality single cell libraries. Collectively, data presented in this chapter confirms the effective optimisation of the scLCM-seq protocol, thereby achieving the preparation of good quality single cell cDNA libraries that can be used for in-depth sequencing.

### 5.3. Results

Previous work has shown that single cell RNA-sequencing can be adapted to diverse input materials and amounts by utilising optimised Smart-seq2 protocols (Nichterwitz et al., 2018, 2016; Picelli et al., 2014, 2013). Nichterwitz et al. demonstrated that it was possible to isolate single motor neurons with small cell soma areas using LCM and couple it with the Smart-seq2 RNA-sequencing technology, thereby yielding good sequencing results (Nichterwitz et al., 2016). However, to date the use of LCM-seq for single cells coupled with Smart-seq2 is not a routinely used and optimised protocol.

In this chapter, I aimed to optimise the use of LCM to collect single motor neurons from the brainstem of young mice with RNA-sequencing protocols utilising Smart-seq2. The goal of this work was to optimise this single cell LCM-sequencing method to investigate the selective vulnerability present in the CN VII (facial nucleus) of the SMN $\Delta$ 7 mouse model. Being able to perform single cell sequencing on a motor neuron population (CN VII) that presents with a highly mixed vulnerability, has the potential to identify distinct cellular signatures of motor neuron subpopulations. In order to investigate whether the single cell LCM-seq workflow could be optimised for the use with single motor neurons from the brainstem, a number of steps needed to be completed and assessed. The strategy for the single motor neuron LCM-sequencing process included 1) brainstem tissue collection from SMN $\Delta$ 7 mice and control animals at three time points (P2 pre-symptomatic, P5 early symptomatic and P9 late symptomatic), 2) single motor neuron collection via LCM from the CN VII, 3) cDNA library preparations from single cells and quality control, and 4) single cell RNA-sequencing and bioinformatics analysis (Figure 5.1).

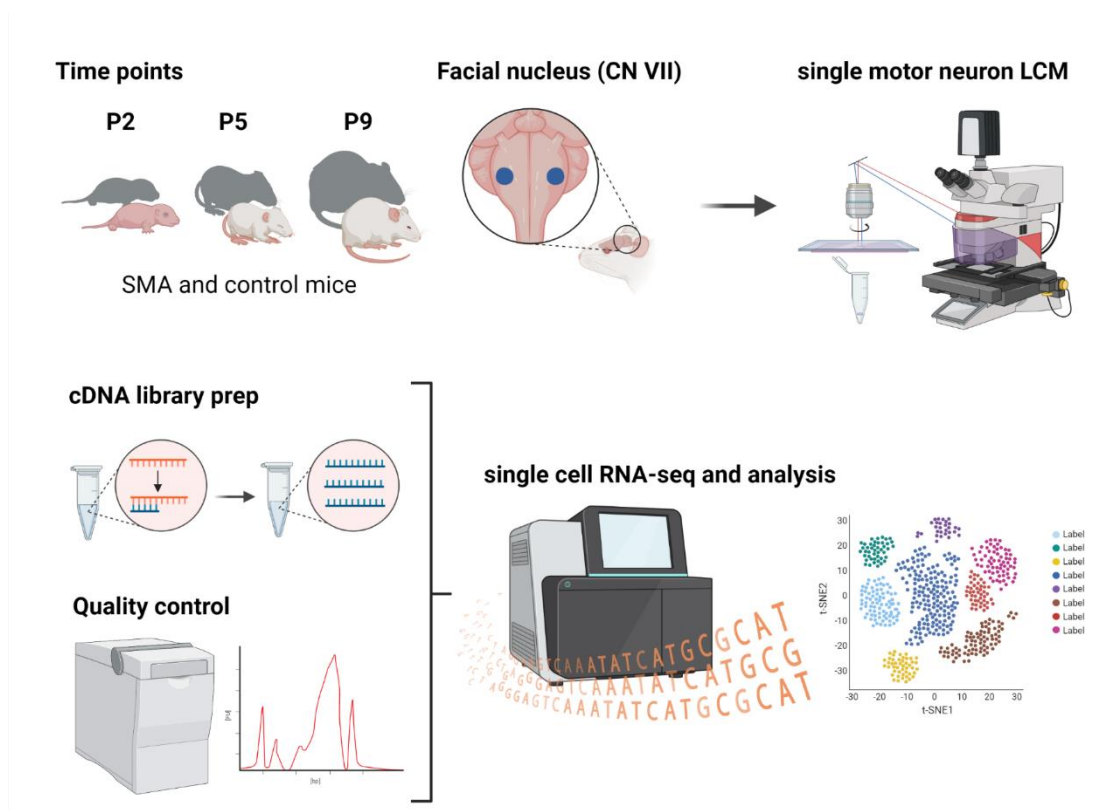


**Figure 5.1: Workflow and strategy of the single motor neuron LCM-sequencing process.** Brainstem tissue was collected from SMN $\Delta$ 7 mice and their control littermates at three time points (P2 pre-symptomatic, P5 early symptomatic and P9 late symptomatic). Single motor neurons from the CN VII were collected by LCM and lysed. The RNA from each collected motor neuron was reverse transcribed and amplified to produce cDNA single cell libraries. Quality control was performed by analysing cDNA concentrations and cDNA molecular size distribution profiles, before sending samples for single cell sequencing and performing bioinformatic analysis.

### 5.3.1. Achieving reliable identification of the CN VII in brain sections from adolescent mice

The first step towards effective isolation of single motor neurons from the CN VII, was to reliably identify the CN VII nucleus in the brainstem of adult mice. The Allen Brain Atlas (<https://mouse.brain-map.org/static/atlas>) is an online atlas that allows to view both sagittal and coronal section of an adult (P56) mouse brain with a great variety of anatomical structures highlighted in each section. This tool allows to track the exact location of a specific anatomical structure on each Nissl-stained section within the brain. The atlas showed that the facial nucleus is located bilaterally at the ventrolateral side of the brainstem, approximately located at the level of the junction between pons and medulla.

To reliably identify the CN VII in the collected tissues from SMN $\Delta$ 7 and control mice, I first replicated the sections shown in the Allen Brain Atlas on our animal tissues (Figure 5.2). On sagittal sections of the adolescent mouse, I was able to locate the position of the facial nucleus, as shown in the online atlas (Figure 5.2A-B). For the use with LCM, it was imperative to quickly and reliably identify the exact location of the CN VII in the collected tissues from SMN $\Delta$ 7 and control mice, to firstly, avoid prolonged sectioning times of samples that could negatively impact the RNA quality, and secondly, to avoid the use of a high number of costly specific glass slides (Carl Zeiss 1.0 PEN membrane slides) used for the tissue collection for LCM. Even though the location of the CN VII could be identified in these sections, it proved difficult to collect every subsequent section of the nucleus. That was because the CN VII was located deeper within the brainstem, making it time consuming and difficult to find the exact section that yielded the beginning of the facial nucleus during sectioning.

According to the Allen Brain Atlas, the facial nucleus could alternatively be identified in coronal sections of the brain, showing its location at roughly the middle of the brainstem on each side of the midline (Figure 5.2C). However, when replicating these sections, the location of the CN VII could not confidently be identified, only being able to roughly identify the correct area where the facial nucleus should be located (Figure 5.2D). The difficulty with identifying the exact location of the facial nucleus in coronal sections seemed to be caused by the lack of clearly identifiable landmarks in the direct proximity of the facial nucleus. The identification of the correct location was further complicated by the fact that the facial nucleus is located at the junction between pons and medulla. When sectioning along a coronal axis, a very large number of sections had to be produced to reach this junction and thus again led to a prolonged sectioning time and the use of a large amount of LCM-suitable glass slides. Therefore, this sectioning approach did not prove sufficient to be used for the planned LCM motor neuron collection.

Murray et al. previously used transverse sectioning of the brainstem for LCM isolation of motor neurons from the facial nucleus (Murray et al., 2015). On transverse sections, starting from the ventral side of the brainstem of the

adolescent mouse, I was able to clearly identify the facial nucleus bilaterally in the brainstem (Figure 5.2E). When sectioning from the ventral side of the brainstem, the facial nucleus was immediately visible after the first few sections. This sectioning approach allowed a very robust identification method for the facial nucleus and a very resource efficient collection of tissue.

Being able to section and identify the facial nucleus in the brainstem of mice represented a vital foundation of this body of work and enabled the start of the tissue collection for this work. Brainstem tissues from experimental SMN $\Delta$ 7 and control littermates were collected at three different time points: P2 as pre-symptomatic, P5 as early symptomatic and P9 as late symptomatic time points. These three time points were chosen to enable the thorough investigation of specific cellular signatures of motor neuron subpopulations present at individual time points or throughout the time course of the disease. In the SMN $\Delta$ 7 mouse model, both homozygous animals (*Smn*<sup>+/+</sup>; *SMN2*; *SMN $\Delta$ 7*; aka WT) and heterozygous animals (*Smn*<sup>+/-</sup>; *SMN2*; *SMN $\Delta$ 7*; aka 'Het') were previously shown to not present with a SMA phenotype, and can be considered as control littermates (Monani et al., 2000). However, to keep the genetic variation within the control group as small as possible, only Het animals were used as littermate controls for this body of work. All tissues were collected from immediately sacrificed mice, embedded, and cryo-sectioned as described in Chapter 2 sections 2.6.1 and 2.6.2. After sectioning, all tissues were shipped on dry ice to the Stockholm University, Sweden for continuation of the single cell LCM sequencing work in Eva Hedlund's laboratory.

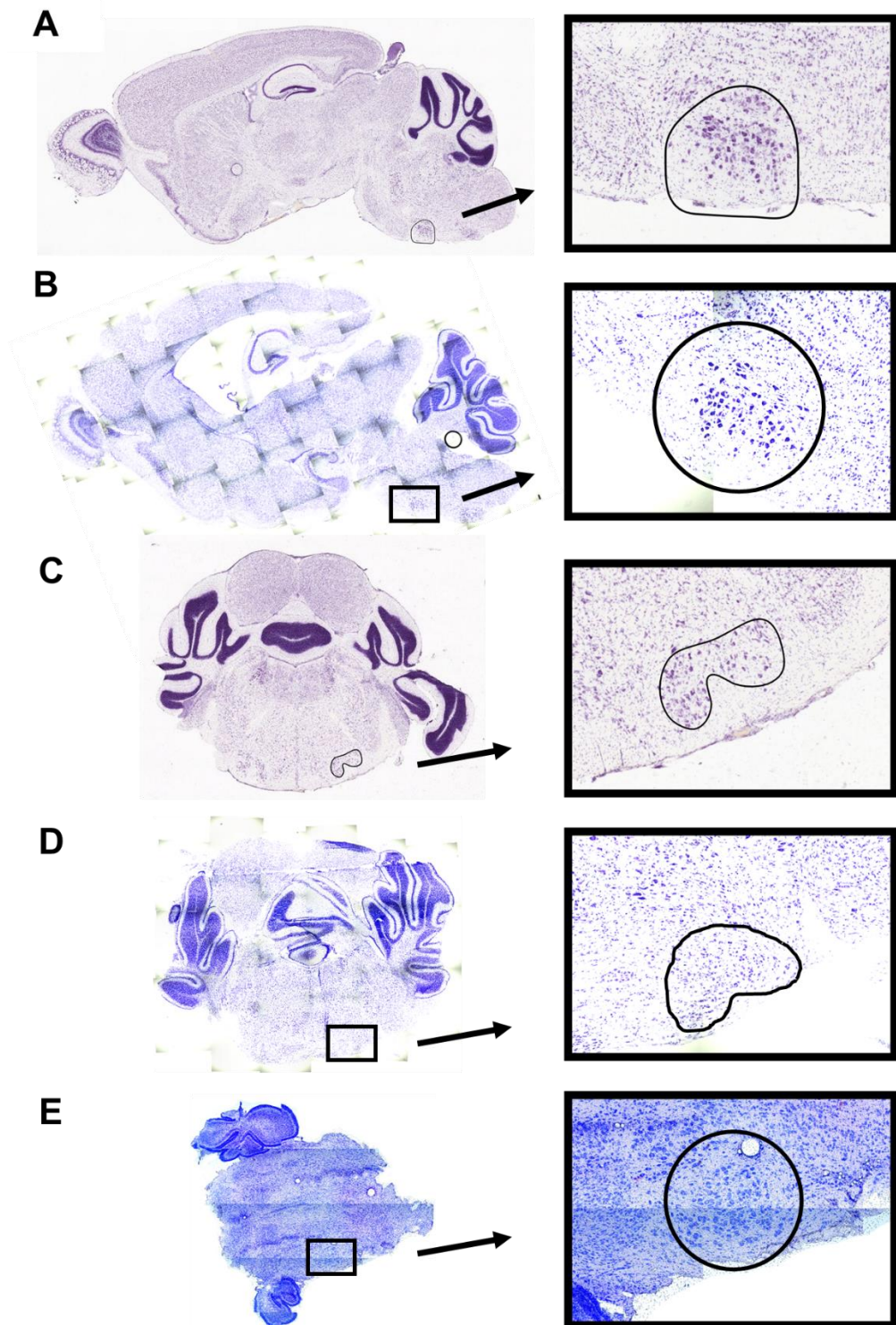


**Figure 5.2: Identification of the CN VII in the brainstem of healthy control mice.** (A) Representative image and indication of the location of the CN VII on a Nissl-stained sagittal section of the adult mouse brain (P56; image taken from the Allen Brain Atlas <https://mouse.brain-map.org/static/atlas>), and (B) my replicated evidence of the CN VII visible in the same location on a Nissl-stained sagittal section of the adolescent mouse brain. (C) Representative image and indication of the location of the CN VII on a Nissl stained coronal section of the adult mouse brain (P56; image taken from the Allen Brain Atlas <https://mouse.brain-map.org/static/atlas>), and (D) possible evidence of the CN VII visible in the same location on a Nissl stained coronal section of the adolescent mouse brain. (E) Representative image and indication of the location of the CN VII on a Nissl-stained transverse section of the brainstem of the adolescent mouse brain from a healthy control mouse.

### **5.3.2. Effective use of LCM for the isolation of single motor neurons from the CN VII of the SMN $\Delta$ 7 mouse model of SMA**

To start the collection of single motor neurons from the CN VII in SMN $\Delta$ 7 and control animals at three different time points, one slide at a time, containing four consecutive brainstem sections, was HistoGene stained to visualise neuronal cells in the tissue. To ensure that only single motor neurons from the CN VII and no other surrounding neuronal cell types were isolated by LCM, specific criteria were set to reliably identify motor neurons. Each collected motor neuron had to have a visible nucleus, was not allowed to overlap with other surrounding neurons, had to be irregular shaped, and had to have a larger than 250 $\mu\text{m}^2$  area. Following these criteria, up to 60 individual motor neurons were collected from consecutive brainstem sections from each animal (Figure 5.3). A cutting outline was drawn as close as possible around each desired motor neuron and cut out of the tissue by LCM, exactly following the drawn outline (Figure 5.3A). Area measurements for each isolated motor neuron were gathered. The shape, size and position of each collected motor neuron allowed mapping to their original anatomical position within the CN VII of the brainstem (see arrow heads in Figure 5.3A-B).

The area measurements of the individually isolated motor neurons from the CN VII from SMN $\Delta$ 7 and control animals at P2, P5 and P9 were analysed (Figure 5.4). The average motor neuron size per animal (averaged from 44-60 single motor neurons per animal) was quantified for all genotypes and time points. The data showed that there was no significant difference in the average motor neuron area between SMN $\Delta$ 7 and control animals at P2 and P5. However, at P9 (late symptomatic time point) the SMN $\Delta$ 7 animals displayed a significant decrease in their average motor neuron area in comparison to their controls (Figure 5.4A). This could be due to the maturation state of the neurons, as the neuronal growth is not necessarily linear with age and body size of the mice. In both control and SMA mice, the average motor neuron area significantly increased between post-natal day 2 and 5 (Figure 5.4B-C). There is no evidence of a further increase of motor neuron area between P5 and P9 in the control and SMA animals. These results are likely due to the rapid and

pronounced postnatal neuronal development that is taking place in these first few days of life.

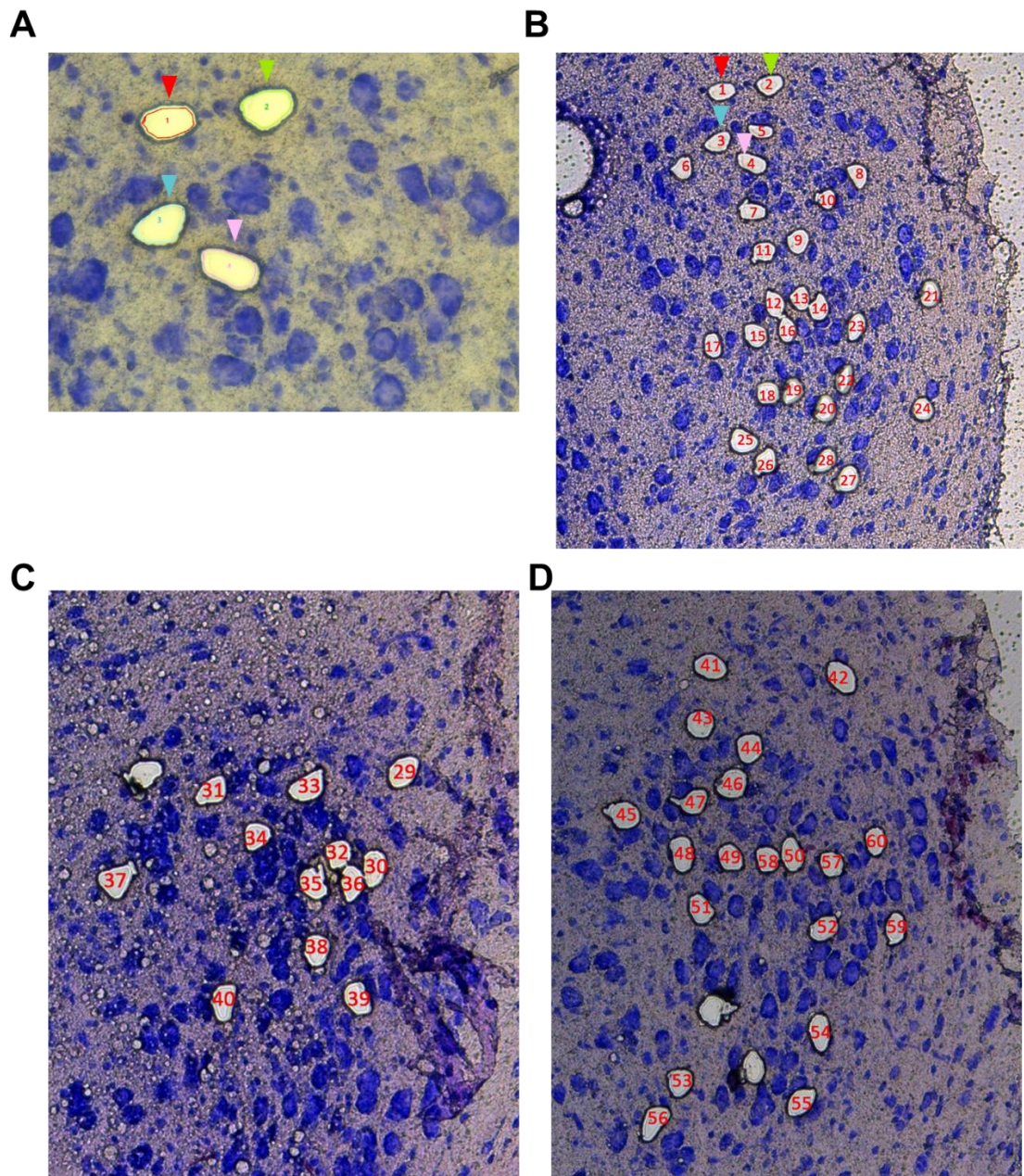


**Figure 5.3: Location mapping of single motor neurons following LCM isolation.** (A) Representative image of four individually collected motor neurons on a HistoGene stained section, indicated by red, green, blue and lilac arrow heads. (B-D) Representative images of three consecutive HistoGene labelled sections from the CN VII of a P5 mouse, depicting how the position of each of the 60 collected single motor neurons of this sample was mapped to their anatomical position within the CN VII.

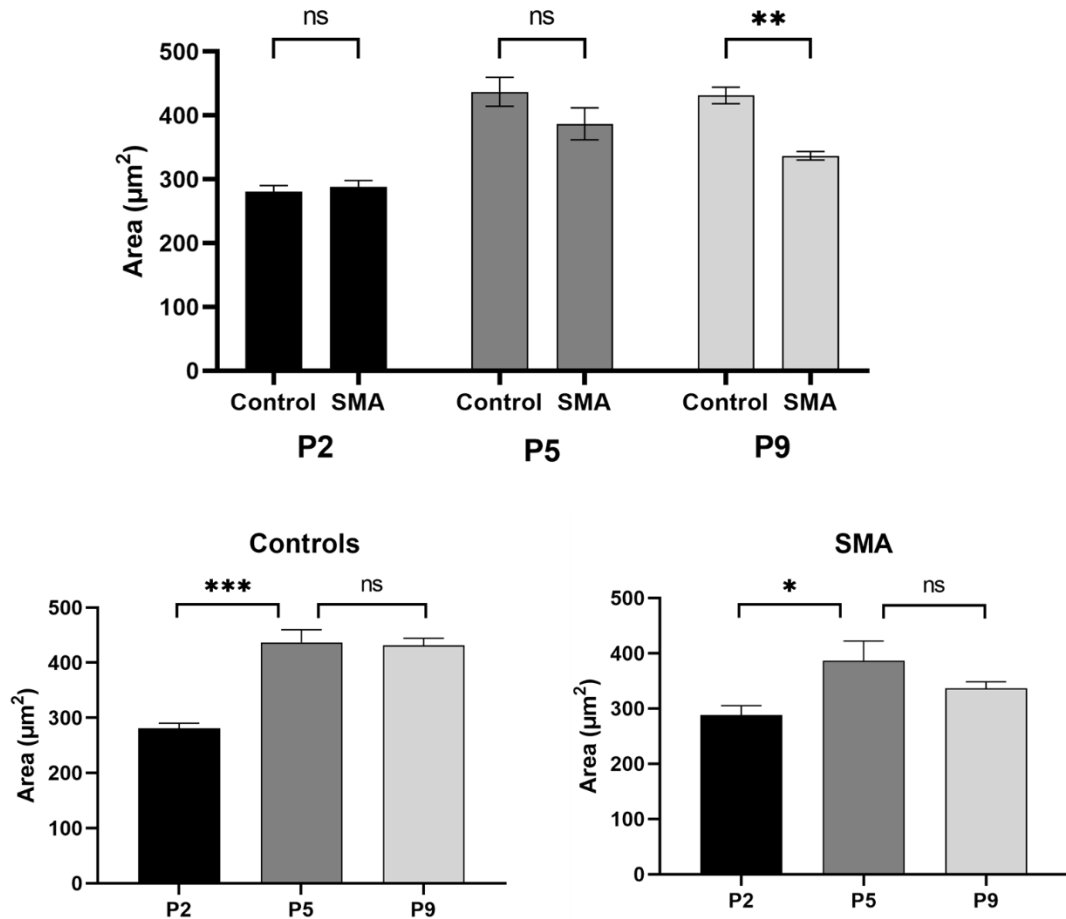


**Figure 5.4: SMN $\Delta$ 7 animals display a significant decrease in average motor neuron area of the CN VII at a late-symptomatic (P9) disease stage in comparison to their controls. (A-C)** Bar charts showing the quantification of the average area size of motor neurons from the CN VII in the SMN $\Delta$ 7 mice compared to their controls at three different time points: pre-symptomatic P2, early symptomatic P5 and late symptomatic P9. Note that there is a significant decrease of the average motor neuron area size from SMN $\Delta$ 7 animals in comparison to their controls at P9. Graphs (B-C) show that there is a significant increase in motor neuron area size between P2 and P5 in both, SMN $\Delta$ 7 and control littermates. This is likely due to the growth and development of the animals during this time. Two-sided ANOVA with Sidak correction (ns=no significance, \* $p \leq 0.05$ , \*\* $p \leq 0.01$  and \*\*\* $p \leq 0.001$ ).  $n=3$  for controls and SMN $\Delta$ 7 mice (average motor neuron size of 44-60 MNs from each individual). Error bars represent mean  $\pm$  SEM.

### **5.3.3. Optimisation of cDNA library preparations of single motor neurons from the CN VII of the SMN $\Delta$ 7 mouse model**

After performing single motor neuron LCM on up to 60 neurons from each SMN $\Delta$ 7 and control animal at three different time points, the goal was to produce good quality cDNA libraries in preparation for single cell RNA-sequencing. Each cell had to undergo several steps to produce cDNA libraries from the RNA of each collected motor neuron. In brief, each cell was lysed directly after collection, first and second strand synthesis was performed to convert RNA to double stranded cDNA, cDNA libraries were purified using SPRI beads, cDNA concentration was quantified, cDNA quality control was performed, libraries were diluted, followed by unique fragmentation and barcoding of each library (tagmentation) and pooling of samples in preparation for single cell RNA-sequencing (Figure 5.5A).

Initially, I prepared cDNA libraries from only 16 of the first collected motor neurons to assess if the utilised cDNA library preparation protocol was working on the LCM collected single motor neurons. The protocol used and optimised for the library preparation of single cells for this body of work was published by Schweingruber et al. (Schweingruber et al., 2023). In this protocol, previous protocols for diverse input materials and amounts (Nichterwitz et al., 2016, 2018; Picelli et al., 2014) were carefully adjusted for the use of single-cell transcriptomics that were obtained from spinal motor neurons derived from human-induced pluripotent stem cells and that were isolated by FACS sorting. Here, single cell lysates were processed to single cell cDNA libraries by performing first and second strand synthesis and cDNA library purification as described in Chapter 2 section 2.6.5. During first strand synthesis, reverse transcription with template switching of the RNA was performed, followed by second strand synthesis and an amplification step to produce double stranded cDNA. After completion of cDNA synthesis, libraries were purified using SPRI beads (Figure 5.5B).

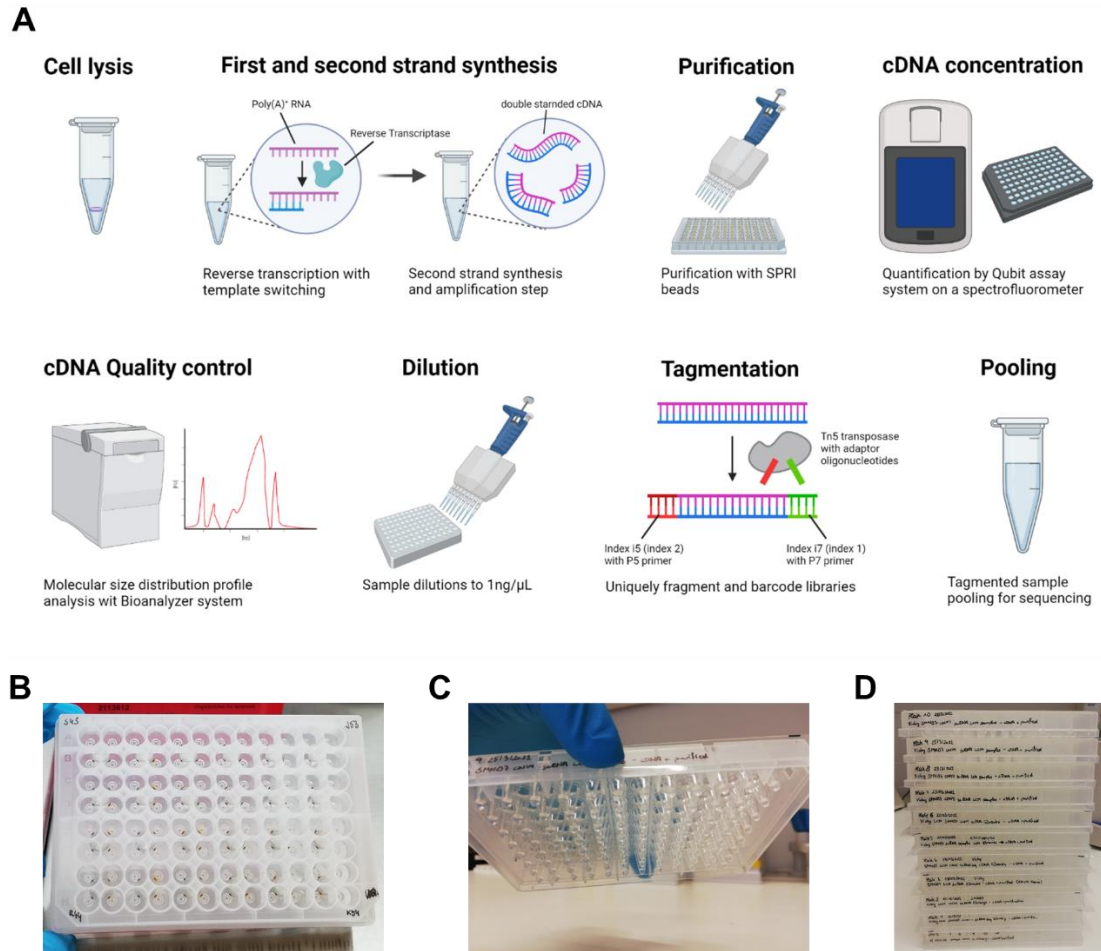


**Figure 5.5: Workflow and strategy of cDNA library preparations from single motor neurons for single cell LCM-sequencing.** (A) Workflow of how to conduct cDNA library preparations of collected single motor neurons from the CN VII in preparation for scLCM-seq. Single motor neurons from the CN VII of SMNΔ7 and control mice were collected by LCM at three different time points (P2 pre-symptomatic, P5 early symptomatic and P9 late symptomatic). Cells were lysed to stop RNA degradation. First strand synthesis was performed by reverse transcription and template switching, followed by a second strand synthesis and amplification of the cDNA. cDNA libraries were purified using SPRI beads and cDNA concentration was quantified with a Qubit assay system using spectrofluorimetry. cDNA quality was assessed by generating molecular size distribution profiles with a Bioanalyzer 2100 system. Libraries were diluted to 1ng/μL and tagmentation was performed. Each cDNA library was uniquely fragmented and barcoded using two specific indices per library (i5 and i7 indices). Tagmented cDNA libraries were pooled in preparation for scLCM-seq. (B) Representative photo of the purification of a 96-well plate of cDNA libraries. (C) Representative photo of a 96-well plate of readily purified cDNA libraries, containing 12μL of cDNA library in each well. (D) Representative photo of all readily prepared and purified libraries in 96-well plates, containing a total of 1051 single motor neuron cDNA libraries.

In order to assess the quality of the produced 16 cDNA libraries and to judge whether the protocol worked effectively for this tissue type and amount, the cDNA concentrations of each library were measured. This was achieved by utilising a Qubit assay system based on a fluorescent dsDNA intercalating dye and recording the fluorescent signal of each sample on a microplate spectrofluorometer. Concentrations of single cell libraries were then calculated as outlined in Chapter 2 section 2.6.6. For single cell libraries, the expected cDNA range is 0.5ng/μL to 5.0ng/μL (Schweingruber et al., 2023). The measured cDNA concentrations from the first 16 produced single cell libraries showed an average of 3.37ng/μL, which was within the expected cDNA concentration range for single cells. Despite showing an expected average cDNA concentration, sample concentrations ranged from -0.77ng/μL to 17.67ng/μL. A negative concentration value could have been the result of this sample consisting of degraded RNA before library preparation. A minimum cDNA concentration requirement of 0.5ng/μL was set for samples to be used for further quality control. This cut off removed one sample, leaving 15 samples for further investigation.

Following measurement of cDNA concentrations, each of the remaining 15 single cell library was diluted to equal cDNA amounts in preparation for further quality control. The quality of each cDNA library was then analysed by visualising the molecular size distribution profile using a Bioanalyzer 2100 system (see section 2.6.6.). The Bioanalyzer system is based on traditional gel electrophoresis principles and allows the quick analysis of quality and composition of DNA samples, by analysing their cDNA amount and fragment size. Therefore, the overall quality of each sample after preamplification and purification could be determined by analysing their measured molecular size distribution profiles (Figure 5.6). The aims of the analysis of molecular size profiles are twofold. Firstly, to detect whether intact mRNA is present and secondly, to visualise a peak in low molecular weight that is indicative of contamination with RT/PCR artifacts such as primer dimers (Schweingruber et al., 2023). Intact mRNA is visualised through the presence of a peak in high molecular weight cDNA (average size of 1.5-2kb), while a wide distribution of low molecular weight cDNA fragments is indicative of degraded mRNA.

Suboptimal or poor cDNA library profiles can be the result of degraded RNA samples or the presence of primer dimers. If the library is prepared with degraded RNA, the profile shows a shift towards shorter DNA fragments with only little amplification of long fragments (Figure 5.6A-B). This type of profile was only visible for a small amount of the initially prepared single cell samples. Around 80% of cDNA libraries in this first attempt showed a suboptimal preamplification profile, consistent with the presence of a high amount of primer dimers. This profile type is characterised by a peak of primer dimers that is higher than the cDNA library (Figure 5.6C-D). These results showed that the preamplification process was not yet optimised, as the collected samples showed obstructive levels of primer dimer.

In an attempt to reduce primer dimers and produce good quality cDNA libraries, some reaction conditions of the library preparation process were adjusted. For the next preparation of a small number of single cell libraries, the used lysis buffer was modified by reducing the amount of added 10 $\mu$ M Smarter\_oligo-dTVN by 50% and not adding ERCC\_Spike-Ins. Further, the amplification cycles of the second strand synthesis reaction which are composed of denaturation, annealing and elongation, were increased from 21 to 24 cycles, in an attempt to provide better preamplification results. Around 67% of molecular size distribution profiles of these optimised cDNA libraries showed good library quality with only small amounts of short DNA fragments (Figure 5.6E-F). These results showed an optimisation of the cDNA library preparation protocol for use with single motor neurons obtained by LCM.

Following the optimisation of the library preparation protocol, the remaining single motor neuron cDNA libraries were prepared. A total of 1049 single motor neuron cDNA libraries were prepared, quality assessed and diluted to 1ng/ $\mu$ L in preparation for single cell RNA sequencing (Figure 5.5C-D). The cDNA concentrations from all samples were measured, showing that the average cDNA concentration of all samples was 2.06ng/ $\mu$ L. This was in line with the expected cDNA range of 0.5ng/ $\mu$ L to 5.0ng/ $\mu$ L for single cell libraries (Schweingruber et al., 2023). Despite showing an expected cDNA concentration upon averaging of all samples, there was a wide range of initial

cDNA concentration present throughout all 1049 libraries, ranging from -1.10ng/μL to 31.08ng/μL. Some cDNA libraries showed a negative concentration value or a very low cDNA concentration which was indicative of problems with these samples. This could have been the result of some samples consisting of degraded RNA before library preparation. A minimum cDNA concentration requirement of 0.5ng/μL was set for samples to be used for future sequencing. This cut off removed 175 samples from the total of 1049 prepped samples, leaving 874 samples for future sequencing. The average cDNA concentration of the remaining 874 samples was 2.41ng/μL, which was within the expected range. These results indicate that 83% of all collected samples had sufficient cDNA concentration to be sequenced.

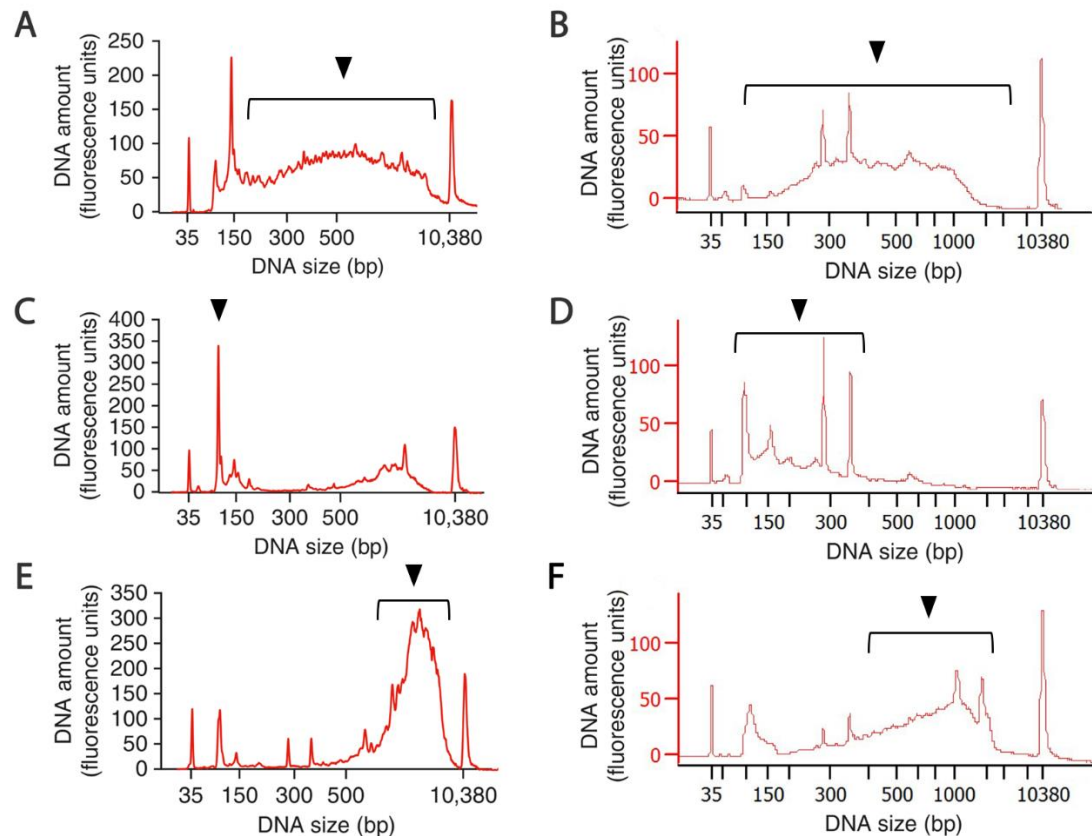


**Figure 5.6: Bioanalyzer profiles of expected profile types compared to libraries from single mouse motor neurons.** (A) Representative bioanalyzer profile showing a wide distribution of cDNA fragments in a single mouse interneuron (graph taken from Picelli et al., 2014), and (B) evidence of the same profile type from one of my single motor neuron libraries from P5. This type of profile is associated with RNA degradation that happened before library preparation. (C) Representative bioanalyzer profile example that show cDNA dominated by primer-dimer from a single human HEK293T cell (graph taken from Picelli et al., 2014), and (D) evidence of the same primer-dimer dominated profile type from one of my single motor neuron libraries from P5. This profile is characterised by only small amounts of amplified cDNA, but a large peak of primer-dimer that was not removed during bead purification. (E) Representative bioanalyzer profiles showing the cDNA distribution of a successful cDNA preamplification from a single mouse MEF cell (graph taken from Picelli et al., 2014), and (F) evidence of a cDNA distribution of a successful cDNA preamplification of one of my single motor neuron libraries from P5. fluorescent unit = amount of cDNA, DNA size = base pairs (fragment size)

#### **5.3.4. MiSeq analysis shows good quality sequencing of cDNA libraries from single CN VII motor neurons of the SMN $\Delta$ 7 mouse model**

After preparing 1049 cDNA libraries from LCM collected single motor neurons from the CN VII, further quality assessment of the produced libraries was required. I investigated whether the sequenced libraries had the potential to yield good quality sequencing data. Despite achieving good results when analysing the molecular size distribution profiles, barcoding and sequencing all collected libraries at a high sequencing depth without a prior sequencing test, would have required a lot of staff resources and posed a very high financial risk. Therefore, a subset of 24 single motor neuron libraries was selected to undergo low depth sequencing on a MiSeq sequencing platform to assess the sequencing quality of the produced libraries.

To ensure the best possible cross-sectional variety of collected samples for sequencing, samples from both SMN $\Delta$ 7 and control animals were chosen, including samples from 16 out of 18 animals that were used for motor neuron collection. Similar amounts of samples from across all three different time points (n=7 for P2, n=9 for P5 and n=8 for P9) were chosen for MiSeq sequencing. Further, a wide range of samples with different initial cDNA concentrations was chosen, ranging from 0.52ng/ $\mu$ L to 12.83ng/ $\mu$ L and averaging at 3.94ng/ $\mu$ L. All 24 chosen samples were uniquely barcoded, fragmented and purified with SPRI beads during the tagmentation process (see Chapter 2 section 2.6.7.) and equal amounts of cDNA from all samples were pooled as final sequencing library for MiSeq. 51-bp single-end sequencing was performed on these 24 samples on a MiSeq sequencing platform.

Upon completed MiSeq sequencing of single motor neuron samples, various quality control parameters were analysed to get a better understanding of the quality of the produced single cell libraries. MiSeq sequencing resulted in a read depth of up to 800,000 reads per sample. An analysis of the FastQ screen of samples at all three different time points (P2, P5 and P9) was conducted (Figure 5.7A). To be able to reach correct conclusion from a sequencing screen, the origin of each sample needs to be determined. The FastQ screen

allows to compare the library of sequences against a set of known sequence databases from different species. This reveals if the composition of the sequenced library matches the expected reference genome, here the mouse genome, or if sources of contaminations are present. Samples can commonly get contaminated during sample collection, through unclean reagents, carry-over from other samples, or sample swaps (Wingett and Andrews, 2018). Depending on the completeness of the gene annotations, it can be expected to see up to 60% of reads uniquely mapped to the reference genome when sequencing 50-bp single-end reads (Picelli et al., 2014). The analysis showed that some libraries showed a high percentage match with the mouse genome, while others showed high percentages of multimapping of other genomes (Figure 5.7A). The additionally mapped genomes of the samples, including the human genome, are all common contamination sources. Prior to performing transcriptional analysis, it will be important to remove the human-derived reads that also align to the reference genome from the mouse to avoid wrong conclusions about biological differences (Wingett and Andrews, 2018).

When looking at the FastQ screen of samples at each individual time point P2, P5 and P9, samples from P5 seem to have the highest overall percentage of reads uniquely mapped to the mouse genome (Figure 5.8). To investigate if a higher percentage match with the reference mouse genome can depend on the initial cDNA concentration of the sequenced sample, a correlation between these parameters was analysed. The results showed that there was no correlation ( $R^2=0.000007822$ ) between the initial cDNA concentration of the sequenced samples and their percentage of uniquely mapped reads to the mouse genome in any of the 24 sequenced samples (Figure 5.7B).

Next, a further quality control parameters of the MiSeq sequenced samples were assessed. A FastQC screen of sequence counts for each MiSeq sample was analysed for all 24 samples. This allows to visualise the percentage of unique and duplicate sequence reads for each sample. Results showed that many sequenced libraries showed between 30% to 60% unique sequence reads (Figure 5.7C). Lastly, to assess the overall sequence quality for each of the 24 samples, the mean quality scores across each base position in the read

was analysed for each sample. The higher the score, the better the quality for each base call. This data can help determine if there were problems during sequencing at the sequencing facility. Lower quality scores towards both ends of the sequence can be expected. The results showed that all sequenced samples consisted of very good quality base calls (green background area on y-axis) across all base positions (Figure 5.7D).

Taken together, the results from the MiSeq sequencing showed convincing evidence that good quality cDNA libraries were produced utilising the novel and optimised single cell LCM-seq method presented in this chapter. Overall, it is concluded that LCM can indeed be used to isolate single motor neurons from frozen brainstem tissues of mice, and that existing single cell sequencing protocols were effectively adapted to work for this input material and amount. Further work to sequence all produced cDNA libraries from SMN $\Delta$ 7 and control animals with high sequencing depth at three different time points would be valuable to investigate remaining research questions.

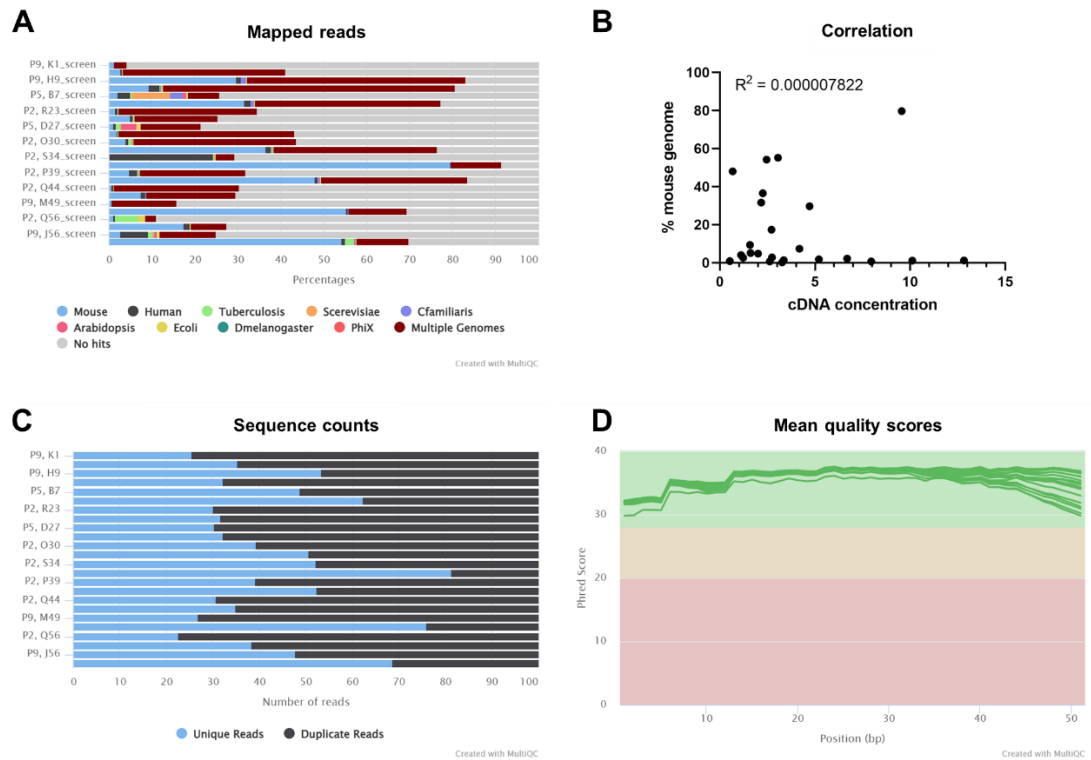


**Figure 5.7: Quality control parameters for MiSeq sequenced single motor neurons from SMN $\Delta$ 7 and control mice. (A)** FastQ screen of MiSeq samples of three different time points (pre-symptomatic P2, early symptomatic P5 and late symptomatic P9). The graph shows the screen of a library of sequences against a set of known sequence databases from different species. This allows to see if the composition of the sequenced library matches the expected genome (here mouse [*Mus musculus*]). Note that, as expected, some libraries show a high percentage match with the mouse genome. **(B)** Scatterplot showing the correlation between the percentage of mouse genome (as shown in A) and the initial cDNA concentration of the sample before sequencing. There is no correlation ( $R^2=0.000007822$ ) between the two variables. Correlation test with Pearson correlation coefficient ( $r=0.002797$ ;  $p=0.9897$ ). **(C)** FastQC screen quality control plot of sequence counts for each MiSeq sample of three different time points. The graph shows the percentage of unique and duplicate sequence reads for each sample. **(D)** Sequence quality histogram showing the mean quality scores across each base position in the read for each MiSeq sample of three different time points. The background of the y-axis shows very good quality calls (green), reasonable quality calls (orange), and poor quality calls (red). Note that the mean quality scores for all sequenced samples are very good.

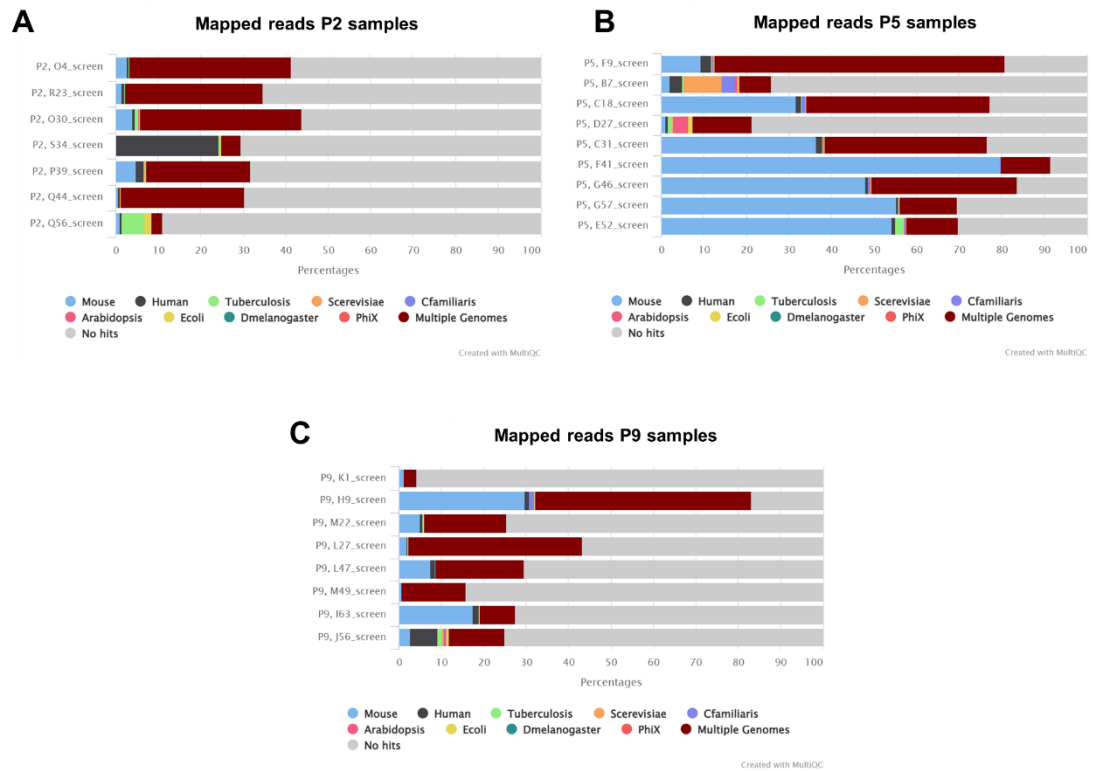


**Figure 5.8: Quality control parameters for MiSeq sequenced single motor neurons from SMN $\Delta$ 7 and control mice at individual time points. (A-C) FastQ screen of MiSeq samples of a pre-symptomatic P2, early symptomatic P5 and late symptomatic P9 time point respectively. The graphs show the screen of a library of sequences against a set of known sequence databases from different species, allowing to see if the composition of the sequenced library matches the expected genome (here mouse [*Mus musculus*]). Note that the generated libraries from time point P5 (B), seem to match the mouse genome the best.**

## **5.4. Discussion**

### **5.4.1. Overview of results**

In this chapter of this thesis, I investigated if existing LCM-seq and single cell RNA-seq protocols could be coupled and effectively optimised for the isolation of single motor neurons from the CN VII of post-mortem tissue in the SMN $\Delta$ 7 mouse model of SMA. I firstly demonstrated that the CN VII nucleus could be reliably identified in transverse sections of the brainstem. This constituted the basis for the LCM isolation of 1049 single motor neurons from SMN $\Delta$ 7 and control littermates. I further showed that the scLCM-seq protocol could be optimised to produce cDNA libraries from all 1049 collected single cells, of which 83% showed sufficient cDNA concentrations to proceed with the experimental strategy. Lastly, I demonstrated on a subset of cDNA libraries that the prepared libraries yielded good quality sequencing results. Initial MiSeq sequencing and subsequent analysis of the subset of samples confirmed the presence of good sequencing quality parameters when sequenced with relatively low sequencing depth (up to 800,00 reads per sample). The results demonstrate that the extension and optimisation of the scLCM-seq protocol was indeed effective, and that the protocol can be utilised for an in-depth sequencing of the remaining produced samples.

### **5.4.2. Future directions and importance of work**

The aim of this body of work was to expand and optimise the scLCM-seq method, allowing the investigation of cellular signature profiles from motor neuron subpopulations from the CN VII in the SMN $\Delta$ 7 mouse model of SMA. While the expansion and optimisation of the scLCM-seq protocol was achieved, thereby producing good quality single cell cDNA libraries for sequencing, the full experimental work for this aim could not yet been completed with the available budget for this PhD project. To complete the full scope of this work as intended, the optimised protocol should be used to perform an in-depth sequencing analysis of all single cell samples from the three collected time points (P2, P5 and P9). To do so, first, the sequencing strategy will need to be decided. A viable decision would be to sequence samples from all three time points in the same sequencing round. With this strategy, samples from all three time points and genotypes will be pooled in

the same Illumina flow cell lane to avoid batch effects due to varying sequencing quality among lanes. This approach would minimise sequencing bias and would possibly provide the best and most comparable data quality. However, sequencing all remaining samples immediately would pose the biggest financial risk, with high tagmentation costs, and with some samples possibly not meeting the desired quality standard and having to be excluded from the final analysis. Similarly, a viable sequencing strategy would be to decide to only immediately sequence samples from P5, as these seemed to show the best match with the mouse genome and the least multimapping to other genomes (Figure 5.8B). This would pose a smaller financial risk but would significantly increase the sequencing bias when separately sequencing individual time points. After deciding on a sequencing approach, good quality cDNA libraries will need to be uniquely barcoded using Nextera XT reagents to enable the sequencing of full-length reads (Chapter 2 section 2.6.7; (Nichterwitz et al., 2018, 2016; Picelli et al., 2014; Schweingruber et al., 2023)). With either above-described sequencing approach, a sequencing platform with sufficient flow cell lanes for the prepared samples and with the capability to perform in-depth sequencing should be chosen. A platform similar to the NovaSeq 6000 that sequences with a 2x100bp read length should be utilised for sequencing of the single cell cDNA libraries.

Following sequencing, a suitable bioinformatics pipeline for the single motor neuron transcriptomics should be chosen to investigate the proposed research question. Importantly, the choice of utilised bioinformatics pipeline has the potential to have a great impact on the quality of the data. Various analysis pipelines can be utilised for this type of data as discussed and demonstrated by Vieth et al. (Vieth et al., 2019). Suitable pipelines can range from standard pipelines, like the one set out by Hwang et al., 2018, to specific pipelines that utilise the *scumi* software package (Ding et al., 2020) or the *scAmp* workflow (Bertolini et al., 2022). No matter which pipeline is chosen, the transcriptional analysis will consist of several steps. First, quality control will be conducted, discarding samples with poor quality or that show minimal uniquely mapped reads with the mouse genome. Next the reads will be aligned to the reference genome and normalisation of the samples will be performed. After

normalisation of the samples, confounding factors will be estimated as the observed read counts are affected by factors like technical noise and biological variable, including batch effects and sample preparation conditions (Hwang et al., 2018). Different motor neuron markers including Islet-1, Islet-2, Mnx1, choline acetyl transferase (ChAT), neurofilament heavy chain (Nefh), and peripherin (Prph) can be identified to confirm that the isolated cells were indeed motor neurons, and the presence of glial markers including Gfap, Mfge8, Aif1, Cx3cr1, Gpr17, Itpr2 and Cnksr3 can be analysed to evaluate the levels of contamination in the isolated samples (Nichterwitz et al., 2020, 2016). Next, different cell types or subpopulations will be identified. This can be achieved by utilising a non-linear dimensionality reduction method, like the t-distributed stochastic neighbour embedding (t-SNE) (van der Maaten and Hinton, 2008). Additional analysis can be conducted to best understand and investigate the differences in cellular signatures from various motor neuron subpopulations of the CN VII. These analysis methods can include clustering of cells and thereby identifying DEGs between different clusters, the inferring of regulatory networks, and reconstruction of the cell hierarchy (Hwang et al., 2018).

The effective expansion and optimisation of the scLCM-seq protocol equips us to conduct an in-depth transcriptional analysis of cell specific signatures of subpopulations from the facial nucleus in the SMN $\Delta$ 7 mouse model of SMA. Effective single cell sequencing enhances our ability to dissect molecular pathways. Instead of showing the mean expression level of a transcripts across a pool of cells, single cell sequencing facilitates the investigation of levels of many transcripts from one cell only. This reveals the interdependence of different transcripts, thus giving a deeper insight into the molecular cascades present within different cell subpopulations. Through the investigation of these specific cell signatures of motor neuron subpopulations, factors could be identified that are involved in motor neuron protection in this mouse model of SMA. Creating refined ways to investigate potential neuroprotective factors, could in turn lead to the identification of important potential pathophysiological targets of the disease. This type of work can facilitate to use identified modifiers of disease as targets for both, new combinational or stand-alone therapeutic strategies.

#### **5.4.3. Limitations of the optimised scLCM-seq protocol**

Despite the effective expansion and optimisation of the scLCM-seq method, there were some limitations to this body of work. As described in this chapter, the optimised protocol facilitated the preparation of good quality cDNA libraries that yielded good quality sequencing results. However, some difficulties remained throughout the experiments. Some samples showed signs of degradation and primer dimer, not all samples showed a sufficient cDNA content to be used for sequencing (Figure 5.6), and some sequenced samples showed a poor match to the reference genome (Figure 5.8). These problems can be due to RNA degradation that could be induced through the shipment of the samples from the UK to Sweden (i.e. through unexpected freeze-thaw cycles), a prolonged collection time of motor neurons with LCM, or a prolonged library preparation time. Further reasons for poor quality samples could be contaminations caused during sample collection (i.e. through the shared use of the LCM system with other groups), error in handling of the samples, or the use of contaminated reagents during library preparation (Wingett and Andrews, 2018). The potential of such complications arising should always be minimised, however, with the novelty of the used scLCM-seq protocol and the simultaneous learning of the experimental methods involved, it is not surprising that some shortages were still present. Going forward, it will be important to utilise a variety of quality controls for the produced data, thereby guaranteeing the exclusion of any poor quality samples from further analysis.

#### **5.4.4. Conclusions**

Overall, the work demonstrates that the optimisation of the single cell LCM-sequencing protocol facilitates the collection and preparation of good quality single cell cDNA libraries from post-mortem mouse tissue. More work is clearly required if we are to shed light on the cellular signature profiles of motor neuron subpopulations from the CN VII motor nucleus in the SMN $\Delta$ 7 mouse model of SMA.

### Conclusions and General Discussion

#### 6.1. Overview of results

Within this thesis, I have presented work that investigated factors and potential transcriptional modifiers that can regulate selective vulnerability of motor neurons and associated skeletal muscles in disease and normal postnatal development in mouse models of spinal muscular atrophy.

In Chapter 3, I investigated the consistency of patterns of selective vulnerability in the four most commonly utilised mouse models of SMA. I conducted a thorough body wide analysis and characterisation of NMJ and motor neuron cell body loss in the *Smn*<sup>2B/-</sup> mouse model at disease end-stage, showing that there is a distinct regional vulnerability pattern present that shows remarkable similarities to the pattern present in SMA patients. I further utilised data from differentially vulnerable muscles from previously published work and supplemented it with my own characterisations to compare the resulting selective vulnerability patterns between four mouse models of SMA. I demonstrated that each mouse models of SMA presented with a distinct pattern of selective vulnerability, while all of them presented with a similar range of synaptic loss. Work in this chapter shows the presence of distinct patterns of vulnerability for each investigated mouse model of SMA and suggests that selective vulnerability can be utilised as a tool for the determination of appropriate experimental designs for specific research questions.

In Chapter 4, I performed two distinct cross-model transcriptional comparisons of differentially vulnerable motor neurons across mouse models of SMA to identify potential neuroprotective modifiers in SMA. For the first comparison, I performed a cross-model transcriptional analysis from previously published data sets to identify a transcriptional signature unique to the resistant motor neurons from the CN VII in the *Smn*<sup>2B/-</sup> mouse model of SMA, showing that an

increase in transcripts associated with ribosomal bioenergetics, vesicle trafficking and neuronal development is correlative with motor neuron protection. Secondly, I performed a cross-model transcriptional analysis from previously published data sets to identify transcripts which show consistent changes in wildtype motor neurons in neuron populations which are known to be vulnerable in SMA mouse models, yielding a refined list of potential neuroprotective modifiers, including PACAP. I tested the effects of PACAP on phenotype and neuropathology *in vivo* in the *Smn*<sup>2B/-</sup> mouse model of SMA, showing that neither a single intracerebroventricular delivery of AAV9-PACAP nor a daily intraperitoneal delivery of purified PACAP protein had a significant effect on phenotype and neuropathology in this model. Overall, in this chapter I provided a list of candidate modifiers and presented thorough workflows to test modifiers for their neuroprotective effects on phenotype and neuropathology *in vivo*.

In Chapter 5, I investigated if existing LCM-sequencing and single cell RNA-sequencing protocols could be coupled and effectively optimised for the isolation of single motor neurons from CN VII of post-mortem tissue in the SMN $\Delta$ 7 mouse model of SMA. In this chapter I presented a workflow that allowed reliable identification of the CN VII motor neurons and effective isolation of more than 1000 single motor neurons from the CN VII from SMN $\Delta$ 7 and control littermates. I further demonstrated that the scLCM-seq protocol could be optimised to produce cDNA libraries with sufficient cDNA concentrations for the experimental workflow and that they yielded good quality sequencing parameter results when undergoing initial MiSeq sequencing. Work presented in this chapter demonstrates an effective extension and optimisation of the scLCM-seq protocol and suggests that this optimised protocol could be utilised for in-depth next generation sequencing of single cells from post-mortem tissues in an array of neurodegenerative diseases.

## **6.2. Importance of findings and future directions**

Therapeutic advances in SMA research over the past few years have produced three approved therapeutics for SMA patients. The main focus of

this research was to develop strategies that restore SMN protein production or increase full-length SMN protein levels in SMA patients. The approved strategies to date include a gene therapy that focuses on the replacement of the mutated *SMN1* gene (Zolgensma) and strategies that modify the aberrant splicing of *SMN2* pre-mRNA through the use of antisense oligonucleotides (Spinraza) or through small splicing-altering molecules (Evrysdi) (as reviewed by Chaytow et al., 2021). Despite these advances in research, none of the current therapies present a cure for the disease. Indeed, children that have been treated with any of these therapies may achieve new motor milestones, but motor deficits remain in treated children and patients continue to present with complex deficits and needs (Audic et al., 2020; Lavie et al., 2021; Matesanz et al., 2019). Combinational therapies have therefore been proposed to be beneficial for individuals with SMA, including non-specific therapies increasing SMN levels, muscle targeting therapies, and targeting cell death pathways (as reviewed by Chaytow et al., 2021; Shorrock et al., 2018). In order to find the most effective stand alone or combinational therapies for patients with all disease severities and demographics, an identification of ever more refined lists of potential candidate modifiers of disease cannot be understated. To identify novel potentially neuroprotective modifiers and therapeutic targets of SMA, we need to have an understanding of the molecular underpinnings of the disease and an evolving understanding of the factors that regulate differential vulnerability in SMA, and that are thereby capable of conferring motor neuron protection. Identified novel modifiers of disease have the potential to function as phenotypic SMN-independent therapies in combination with previously approved SMN-dependent therapeutics. Neuroprotective transcripts could be utilised as combinational therapeutic strategies in different ways, including by overexpression or suppression of the transcript via a viral vector, or through the manufacturing of a small molecule drug that modifies the splicing of the relevant RNA to alter their protein expression. Targeting pathways that are associated with novel modifiers in combination with SMN-restoring therapies could increase the therapeutic benefit to SMA patients.

In this thesis, I have investigated patterns of selective vulnerability from the most commonly utilised mouse models of SMA and have demonstrated that there are vast differences between these patterns. This knowledge of different selective vulnerability patterns can be utilised as a tool to gain novel insights into factors that confer motor neuron protection. For example, I utilised selective vulnerability patterns to identify the facial (CN VII) nucleus as a region of interest for the experimental design of a cross-model transcriptional study. I utilised differentially vulnerable motor neuron populations from two different mouse models of SMA to identify a unique signature of resistant facial nucleus motor neurons, thereby presenting groups of transcripts that have the potential to indeed be important modifiers of the disease. The presented transcriptional analyses in Chapter 4 of this thesis, demonstrates effective workflows of utilising selective vulnerability of motor neurons to identify lists of potential neuroprotective modifiers in a neurodegenerative disease that is thought to be a 'die-back' disorder. The here identified transcripts could be consistent with convergent protective pathways that are active in dying-back pathologies, like ALS and Huntington's disease, and could facilitate the identification of common targets of neuroprotection which could be protective across different neurodegenerative diseases where dying-back pathology is a feature of the disease.

Through the utilisation of the knowledge of selective vulnerability, I designed a further transcriptional comparison of motor neuron populations that are differentially vulnerable in SMA mouse models. By identifying common directional changes in those motor neuron populations from wildtype mice, I identified a refined list of strong candidate modifiers for motor neuron protection. The effectiveness of this experimental approach has previously been demonstrated by combining four different screens of neurologically normal mice, rats and humans to identify neuroprotective modifiers of SMA (Kline et al., 2017). Following this approach by comparing screens from different neurologically normal mice, promises that the yielded refined list of candidate modifiers includes some potential targets for combinational therapies. I subsequently presented two workflows for testing one of the identified modifiers *in vivo*: a workflow for daily IP injected purified protein and

a workflow for a single ICV injection of a AAV9 viral vector. By applying these workflows, I tested the hypothesis that an increase in PACAP levels can reduce synaptic loss and alleviate the presented motor phenotype of an intermediate mouse model of SMA. Although the data from the PACAP work is negative, this underlines the importance of testing potential modifiers in different pre-clinical models to evaluate their actual potential for neuroprotective properties. While PACAP does not appear to be a regulator of selective vulnerability in SMA, the workflows implemented in Chapter 4 have described two strategies to investigate neuroprotective effects on phenotype and neuropathology in mouse models of SMA. The workflows presented here, include experiments to assess aspects of motor performance and neuropathology in a neurodegenerative disease. The workflows could be extended to include different types of motor performance tests and assessment of protein levels of the overexpressed modifier. With these additions, the presented workflows could be transferred into research of other neurodegenerative disorders that are known to present with motor deficits and neuropathology in mouse models of the disease. An example could be to utilise this workflow to test potential neuroprotective modifiers in the SOD1 mouse model of ALS or the R6/2 mouse model of Huntington's disease.

In a next step, it would be interesting to evaluate the neuroprotective properties of the remaining identified candidate modifiers from the refined list identified in Chapter 4. I would propose to utilise a different *in vivo* model for initial evaluation of the efficacy of these modifiers in animal models of SMA. To quickly and effectively validate if any of the remaining transcripts can modify neurodegenerative pathways, the expression of candidate genes could be increased or decreased in a drosophila model of SMA. This approach has previously been utilised to quickly validate modifier candidates after a multi-model transcriptional analysis (Kline et al., 2017). The validation of several identified genes as modifiers of disease in a drosophila model, further verified the multi-model transcriptional profiling approach utilised in their study (Kline et al., 2017) and the subsequently utilised similar approach in this thesis. Following, I propose, that tested transcripts which displayed an ability to modify the phenotype in drosophila, could in a next step then be subjected to *in vitro*

assessment in cell culture models of SMA, like iPSC-derived motor neurons. Testing potential modifiers of disease in iPSC-derived tissues, provides the opportunity to investigate their neuroprotective effects on patient-specific cells with underlying SMA pathology, instead of animal models with SMA-like genetic backgrounds. Alternatively, to the *in vitro* testing in cell culture models, transcripts that modified the drosophila phenotype could subsequently be subjected to extensive *in vivo* testing in a mammalian model of SMA. Here, the proposed AAV9 dosing workflow from Chapter 4 could be utilised and extended to firstly confirm the expression of the vector derived transgene, by analysing its protein levels in the CNS by western blot analysis. Following, the workflow can be extended to include several motor performance tests and additional neuropathology analyses. The testing of promising modifiers in a mammalian model of SMA, offers widespread options for the in-depth validation of actual neuroprotective properties of candidate modifiers. This exemplifies how identified candidate modifiers can be tested in *in vivo* models to identify their actual therapeutic value and showcase them as novel targets for combinational therapy approaches.

I further utilised the acquired knowledge about selective vulnerability patterns in mouse models of SMA, to identify a highly heterogeneous population of motor neurons in a severe mouse model of SMA - the facial nucleus. Utilising this heterogeneous motor neuron population, facilitates the investigation of specific cellular signatures that are present within resistant and vulnerable subpopulations of the CN VII, thereby gaining novel insights into unique protective cellular signatures. In order to investigate the cellular signatures of these motor neuron subpopulations, I extended and optimised existing LCM-seq (Nichterwitz et al., 2018, 2016) and scRNA-seq (Schweingruber et al., 2023) protocols to develop an effective workflow for the isolation and cDNA library preparation of single motor neurons from post-mortem tissue. The workflow of the optimised scLCM-seq protocol implemented in Chapter 5, describes an effective workflow to prepare cDNA libraries from single motor neurons for subsequent sequencing. By utilising this workflow, specific cellular signature profiles that can confer motor neuron protection can be identified and subsequently utilised to advance research on novel therapeutic targets in

SMA. The implemented workflow for the optimisation of a scLCM-seq protocol can further be utilised in various neurodegenerative disorders and is not specific to SMA research. The presented protocol could also be utilised in other neurodegenerative diseases where differential vulnerability of specific neuron populations is a known feature of the disease, or where a specific cell population is assumed to be highly heterogeneous. One example could be to utilise this scLCMseq protocol on vulnerable striatal or hippocampal neuron populations from mouse models of Huntington's disease for the identification of specific cellular signatures that might render these subpopulations more vulnerable.

### **6.3. Concluding remarks**

This work has demonstrated that selective vulnerability presents an invaluable tool to investigate underlying cellular mechanisms of SMA in mouse models of the disease. I have demonstrated that selective vulnerability can be utilised to provide novel insights into the mechanistic foundations of motor neuron protection in SMA mouse models of different severities and underlying genetics. Utilising patterns of selective vulnerability and differentially vulnerable motor neuron populations from wildtype and SMA mice across mouse models of the disease, has allowed me to demonstrate refined lists of neuroprotective candidate modifiers and enabled me to develop workflows of testing these modifiers, as well as to optimise a next generation sequencing protocol to further investigate specific cellular signatures of neuronal subpopulations. I would like to conclude this thesis by emphasising that an in-depth understanding of the molecular underpinnings that regulate selective vulnerability in SMA is invaluable and cannot be understated. By identifying factors that confer motor neuron protection in SMA, we make towards reversing or preventing neurodegeneration by identifying novel targets for therapeutic strategies to battle SMA.

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## Appendices

### 7.1. Appendix 1: Skeletal Muscle paper – first author

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Skeletal Muscle

#### RESEARCH

#### Open Access

## Mouse models of SMA show divergent patterns of neuronal vulnerability and resilience



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### Abstract

**Background:** Spinal muscular atrophy (SMA) is a form of motor neuron disease affecting primarily children characterised by the loss of lower motor neurons (MNs). Breakdown of the neuromuscular junctions (NMJs) is an early pathological event in SMA. However, not all motor neurons are equally vulnerable, with some populations being lost early in the disease while others remain intact at the disease end-stage. A thorough understanding of the basis of this selective vulnerability will give critical insight into the factors which prohibit pathology in certain motor neuron populations and consequently help identify novel neuroprotective strategies.

**Methods:** To retrieve a comprehensive understanding of motor neuron susceptibility in SMA, we mapped NMJ pathology in 20 muscles from the *Smn*<sup>2B/-</sup> SMA mouse model and cross-compared these data with published data from three other commonly used mouse models. To gain insight into the molecular mechanisms regulating selective resilience and vulnerability, we analysed published RNA sequencing data acquired from differentially vulnerable motor neurons from two different SMA mouse models.

**Results:** In the *Smn*<sup>2B/-</sup> mouse model of SMA, we identified substantial NMJ loss in the muscles from the core, neck, proximal hind limbs and proximal forelimbs, with a marked reduction in denervation in the distal limbs and head. Motor neuron cell body loss was greater at T5 and T11 compared with L5. We subsequently show that although widespread denervation is observed in each SMA mouse model (with the notable exception of the Taiwanese model), all models have a distinct pattern of selective vulnerability. A comparison of previously published data sets reveals novel transcripts upregulated with a disease in selectively resistant motor neurons, including genes involved in axonal transport, RNA processing and mitochondrial bioenergetics.

**Conclusions:** Our work demonstrates that the *Smn*<sup>2B/-</sup> mouse model shows a pattern of selective vulnerability which bears resemblance to the regional pathology observed in SMA patients. We found drastic differences in patterns of selective vulnerability across the four SMA mouse models, which is critical to consider during experimental design. We also identified transcript groups that potentially contribute to the protection of certain motor neurons in SMA mouse models.

**Keywords:** Spinal muscular atrophy, Mouse model, Selective vulnerability, Neuromuscular junction, NMJ, Motor neuron, Degeneration, Transcriptional analysis, Gene ontology

### Background

Spinal muscular atrophy (SMA) is a form of motor neuron disease affecting primarily children that is caused by mutations and deletions within the survival motor neuron 1 (*SMN1*) gene [1]. The reduced levels of survival motor neuron (SMN) protein cause progressive loss of lower motor neurons and atrophy of associated skeletal

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muscles [1–3]. A second partially functional copy of this gene (termed *SMN2*) can exist in varying copy numbers and is an important modifier of disease severity [4, 5]. Consequently, SMA can be classified into 4 subtypes based upon the age of onset and expected prognosis. The most severe and common form of SMA (type 1) has an onset of 6 months or earlier and has—if untreated—a life expectancy of 2 years without significant respiratory support. Three treatments have now been approved for patients with SMA [6–11]. Despite the significant benefits that these can confer to patients, impactful motor deficits can remain, even when treatment is administered pre-symptomatically [12, 13]. In order to optimise treatment for all patients, it is imperative that we maintain momentum to understand the fundamental biology underlying SMA.

The central clinical manifestations of SMA are driven by the loss of lower motor neurons. Here, the synapses between motor neurons and muscles, neuromuscular junctions (NMJs), are early and important pathological targets [14], with NMJ loss coinciding with the onset of cell death pathways at the soma, but preceding a quantifiable loss of motor neurons [15, 16]. A wide range of morphological and functional defects of NMJs have previously been reported in mouse models of the disease [14, 17–20]. Although the sequential breakdown of motor neurons is relatively well defined, it has become apparent that not all motor neurons are uniformly vulnerable. Indeed, it has long been recognised that severe SMA patients have more profound weakness in the core and proximal limb muscles, with a comparative sparing of the muscles of the distal limbs, hands, feet and face [21]. Indeed, a more recent study examining muscle function using hammer-smith motor function scales found that muscle location was a predictor of relative weakness, wherein weakness was more profound in proximal limb muscles compared to distal limb muscles [22]. In type 3 SMA patients, patterns of vulnerability are once again highly consistent, with increased vulnerability of the triceps, iliopsoas, thigh adductors, and quadriceps [23].

All SMA mouse models examined thus far show dramatic intermuscular variability in the levels of NMJ loss observed [14, 20, 24–29]. This has been characterised in different mouse models of SMA, including the *Smn*<sup>-/-</sup>;*SMN2* (life expectancy 6 days) [14, 24, 25], *SMN* <sup>$\Delta 7/\Delta 7$</sup> ;*SMN2* aka Taiwanese (life expectancy 8–10 days) [29] and *Smn*<sup>-/-</sup>;*SMN2*;*SMN* <sup>$\Delta 7/\Delta 7$</sup>  aka *SMN* $\Delta 7$  (life expectancy 13 days) [14, 20, 28]. In addition, there is the *Smn*<sup>2B/-</sup> mouse model, created through the mutation of a splice enhancing site in the murine *Smn* gene [30, 31]. Its life expectancy and severity varies depending on the genetic background [26, 27, 30–32], but in our hands, it has a phenotypic onset of 10 days and a

life expectancy of 18 days [15]. Although NMJ pathology and selective vulnerability are evident in this model, only a small number of muscles have thus far been characterised [15, 27].

The reasons for differential vulnerability among motor neurons in SMA are currently unclear, but it is increasingly important that we understand the patterns and investigate their basis. Indeed, it is well established that early administration of *Smn*-dependant therapeutics confers the greatest benefit to patients, with reductions in efficacy observed as treatment is delayed [33]. We have recently shown the recovery of neuromuscular connectivity is proportionate to the time of treatment, but is also highly dependent upon the relative vulnerability of the muscle [34]. Electrophysiology in patients has shown that treatment can induce an increase in the number of active motor units which relate to gains in motor function [35]. Early findings would suggest that the gains in motor function are proportionate to the relative vulnerability of the muscle, and the time period since the motor function was lost. It is therefore critical to profile this selective vulnerability in experimental models and thus provide a platform for the development of future combinatorial therapeutics which will target the most vulnerable motor units.

The phenomenon of differential resilience also provides an opportunity to investigate mechanisms that drive motor neuron protection, which can reveal disease modifiers. This approach has been used to identify modifiers of motor neuron pathology in ALS. Here, motor neurons with differential susceptibility in ALS were isolated from post mortem tissues of neurologically normal mice, rats or humans and subjected to global transcriptome analysis. This led to the identification of a number of important modifiers, including IGF-II [36], GABA and glutamate receptors [37], MMP-9 [38] and synaptotagmin 13 [39]. In SMA, the same approach led to the important identification of DNA repair transcripts [27] and Pdgk1 [40] as potential disease modifiers. In the former study, a four-way comparison of motor neurons which were vulnerable or resistant in wildtype and *Smn*<sup>2B/-</sup> mice facilitated the identification of transcripts associated with a reduced *Smn* level and those specifically involved in the motor unit breakdown. A longitudinal study of transcriptional profiling on discrete neuronal populations at pre-, early- and late-symptomatic timepoints from wildtype and *SMN* $\Delta 7$  mice further identified potential modifiers of disease, including Gdf-11 [41]. Gene ontology and protein network analysis revealed unique disease-adaptation mechanisms present in resistant ocular motor neurons that could be responsible for their sparing. These motor neurons were found to

upregulate transcripts associated with modulation of neurotransmitter release, neuronal survival and protection from oxidative stress. The study revealed cell-specific changes that present promising targets for the protection of vulnerable motor neurons [41]. Combining transcriptional screens on motor neurons which are differentially vulnerable in ALS and SMA can significantly refine the list of potential modifiers and lead to the identification of SNCA [42] and STMN1 [42, 43] as promising neuroprotective modifiers. These findings prove that comparing selectively resilient with vulnerable motor neurons can lead to the discovery of mechanisms that drive protection. It also demonstrates that combining findings from different disease models increases the likelihood of effective identification of promising disease modifiers. Uncovering the similarities and differences in selective vulnerability patterns across models can provide a new platform to uncover the mechanism driving motor neuron resistance and susceptibility.

In this study, we performed a thorough body-wide analysis of selectively vulnerable patterns in the *Smn*<sup>2B/-</sup> mouse model. We demonstrate that the *Smn*<sup>2B/-</sup> mouse model displays a unique pattern of selective vulnerability, wherein the muscles from the core, proximal forelimbs and proximal hindlimbs display increased NMJ loss. We further show that this peripheral pathology correlates with an increased loss of motor neurons in the associated spinal cord segments. This regional pattern of vulnerability, where the proximal muscles are more vulnerable than the distal muscles, shows similarities to the patterns of vulnerability observed in patients [21–23]. We subsequently assembled previously published results from selectively vulnerable muscle analyses from the *Smn*<sup>-/-</sup>; *SMN2*, Taiwanese and *SMNΔ7* mouse models and used a seven-point colour-coded classification tool to compare patterns of selective vulnerability within and between models. While most models (with the notable exception of the Taiwanese mouse model) displayed denervation in a range of muscles, they each showed unique patterns of selective vulnerability. For example, NMJs of facial motor neurons were highly vulnerable in the *SMNΔ7* mouse model, while completely protected in the *Smn*<sup>2B/-</sup> mouse model. To further identify groups of transcripts that have the potential to drive motor neuron protection, we performed cross-model comparative bioinformatics analysis and enrichment analysis utilising published RNA-seq data on facial motor neurons from *Smn*<sup>2B/-</sup> and *SMNΔ7* mice. We demonstrate that an increase in transcripts associated with protein localisation to cell periphery and ribonucleoprotein complex biogenesis correlated with protection from

degeneration. We propose that these transcript clusters could contribute to the protection of motor neurons in SMA mouse models.

## Materials and methods

### Mouse maintenance

*Smn*<sup>2B/2B</sup> mice [22; C57Bl/6J congenic background] were interbred with *Smn*<sup>+/-</sup> (Jackson Laboratories strain formerly 010921, now 006214, C57Bl/6J congenic background) to obtain *Smn*<sup>2B/+</sup> control mice and *Smn*<sup>2B/-</sup> experimental mice. *Smn*<sup>+/-</sup>; *SMN2*<sup>copy</sup>; *SMNΔ7*<sup>copy</sup> (Jackson strain 005025, FVB congenic background) mice were interbred to produce *Smn*<sup>-/-</sup>; *SMN2*<sup>copy</sup>; *SMNΔ7*<sup>copy</sup> (aka *SMNΔ7*) mice. All mice were maintained on a 12-h light/dark cycle under pathogen-free conditions within animal facilities of the University of Edinburgh. All procedures were performed in accordance with the UK Home Office.

### Neuromuscular junction labelling and quantification

The muscles were immediately dissected from recently sacrificed mice and were fixed in 4% paraformaldehyde (PFA; Electron Microscopy Science) in PBS for 15 min. All muscles were microdissected post-fixation and permeabilised in 2% Triton X-100 (Sigma; PTB) in PBS for 30 min at room temperature. They were blocked in 4% bovine serum albumin (BSA)/1% Triton X-100 in PBS for 30 min followed by overnight incubation in primary antibodies (Neurofilament [NF; 2H3]; 1:50 and synaptic vesicle protein 2 [SV2]; 1:100 from Developmental Studies Hybridoma Bank) in blocking solution at 4 °C. Tetramethylrhodamine-conjugated α-bungarotoxin (BTX; 1:250; Thermo Fisher) in PBS was applied for 60 min at room temperature followed by PBS washes. The muscles were incubated in secondary antibody (Alexa Fluor 488 Rabbit anti-Mouse; Jackson ImmunoResearch Laboratories; 1:250) in PBS for 3–4 h in the dark at room temperature and washed with PBS. The muscles were whole-mounted on glass slides (Thermo Fisher) using Mowiol® (Calbiochem) and cover-slipped.

Standard protocols for NMJ quantification were applied [14, 26–28, 42–45]. NMJ occupancy was quantified manually using a Leica-inverted fluorescent microscope at 40x magnification using filters that allow separate and simultaneous visualisation of green (NF, SV2) and red channels (BTX). All quantifications were undertaken blinded to the genotype of the muscle. A minimum of 100 endplates in three or more fields of view per muscle were quantified. The fields of view were chosen randomly across the muscle using the BTX staining. The analysed endplates were classified as vacant, partially occupied (where a pre-synaptic terminal only partially covers an AChR cluster) and fully occupied (see Fig. 1A

for example). Representative example images are projections of z-stacks obtained by a Leica SP8 confocal microscope at  $\times 63$  magnification.

#### Motor neuron labelling and quantification

The spinal cords were immediately dissected from recently sacrificed mice and fixed overnight in 4% para-formaldehyde (PFA; Electron Microscopy Science) in PBS. They were immersed in 30% sucrose in PBS for 48 h prior to embedding in 50% optimal cutting temperature compound (OCT)/15% sucrose in PBS. Spinal cord segments T5, T11 and L5 were transverse sectioned at 10  $\mu$ M. Every tenth section was collected and labelled with Nissl/ChAT.

Sections were permeabilised in 0.3% Triton X-100 in PBS for 30 min and blocked in 0.3% Triton X-100/4% bovine serum albumin (BSA) in PBS for 60 min. Sections were incubated for three nights in ChAT (goat anti-choline acetyl-transferase; Merck Millipore; 1:100) primary antibody in a blocking solution at 4 °C. Slides were washed in 0.2% Triton X-100 in PBS and incubated in secondary antibody (AlexaFluor 555 Donkey anti-Goat; Life Technologies; 1:250) in a blocking solution for 2 h at room temperature. Sections were counterstained with DAPI (Life Technologies; 1:1000) and NeuroTrace® (Nissl; Life Technologies; 1:100) and mounted in Mowiol® (Calbiochem).

The total number of motor neuron cell bodies was quantified by fluorescent microscopy at 20x magnification using a Leica-inverted fluorescent microscope with filters that allow separate and simultaneous visualisation of green (Nissl) and red (ChAT) channels (example images shown in magenta green). All quantifications were undertaken blinded to the genotype of the sections. The number of ChAT-positive motor neurons in the ventral horn of the spinal cord was quantified bilaterally in every tenth section. Values that were missing due to sectioning artifacts were statistically imputed using the *mice: Multivariate Imputation by Chained Equations in R* [46] package in R studio. The values

were assumed as missing at random (MAR) and were imputed using a predictive mean matching (PMM) imputation model. Five different values were predicted for each missing data point. The average from these five predicted values was used as the final imputed value for the following statistical analysis of the total number of motor neuron cell bodies in each spinal cord segment. Approximately, 12% of all values were imputed. Images were taken on a Leica DM8 fluorescent microscope at 20x magnification.

#### Data acquisition and analysis for multi-model comparison of selective vulnerability patterns

Data on neuromuscular junction pathology in a range of muscles was obtained from previously published studies in four mouse models of SMA. Studies detailing NMJ pathology in *Smn*<sup>-/-</sup>;*SMN2*<sup>2copy</sup> (herein referred to as *Smn*<sup>-/-</sup>;*SMN2*) [14, 24, 25], *Smn* <sup>$\Delta 7/\Delta 7$</sup> ;*SMN2* aka Taiwanese [29], *SMN* $\Delta 7$  [14, 20, 28] and *Smn*<sup>28/-</sup> [27] mouse models were utilised. Graphs showing the percentage of fully occupied endplates were exported from studies into Adobe Photoshop (version 21.2.0). A grid overlay was aligned to each graph's axes to allow exact measurements of the mean percentage of fully occupied motor endplates.

Following the acquisition of the data, results were pooled by mouse model and the muscles were allocated to seven classification categories according to their percentage of fully occupied endplates at the end-stage of the disease. Each classification category was assigned a different colour (ranging from green, representing the muscles with minimal synaptic loss, to magenta representing the muscles with large amounts of NMJ loss) for representation in figures. The first six categories each include the muscles with percentages of fully occupied endplates within a 10% range, forming categories from 100 to 41% fully occupied endplates. The last category includes all the muscles that have 40% or

(See figure on next page.)

**Fig. 1** NMJ loss is more severe in muscles innervated by cervical, thoracic and upper lumbar regions of the spinal cord in *Smn*<sup>28/-</sup> mouse model at P16. **A–C** Representative confocal images showing NMJs labelled with antibodies against NF (green) and SV2 (green) and BTX (magenta) staining in a control muscle (*Smn*<sup>28/-</sup>; gracilis), a vulnerable *Smn*<sup>28/-</sup> muscle (transversus abdominis) and a resistant *Smn*<sup>28/-</sup> muscle (lumbicals), respectively. Pink arrowheads indicate fully occupied endplates, yellow arrowheads show partially innervated endplates, and blue arrowheads indicate vacant endplates. Scale bar: 25  $\mu$ m. **D** Bar charts showing the quantification of percentage of full, partial and vacant endplates in *Smn*<sup>28/-</sup> mice in all investigated muscles. The muscles are displayed in order of their innervation from the cranial nerves and spinal cord segments, starting at the top with the muscles innervated by cranial nerves V (CN V) and VI (CN VI), followed by the muscles innervated from nerves arising from the cervical spinal cord and ending at the bottom with the muscles innervated by nerves arising from the lumbar spinal cord. The spinal cord segments that supply nerve innervation to the analysed muscles are indicated beside the respective bars. A high level of NMJ loss was visible across the muscles that are innervated by the nerves from cervical, thoracic and upper lumbar spinal cord segments. This pattern suggests that NMJ pathology starts at the centre of the spinal cord.  $n=3$  in IC and  $n=4$  in all other muscles for *Smn*<sup>28/-</sup> mice. Error bars represent mean  $\pm$  SEM. **E** Schematic diagram of the relationship between location of spinal cord segments and the anatomical location of the muscles innervated by the nerves arising from the indicated spinal cord segments, starting with the head muscles. Colours are for illustrative purposes only

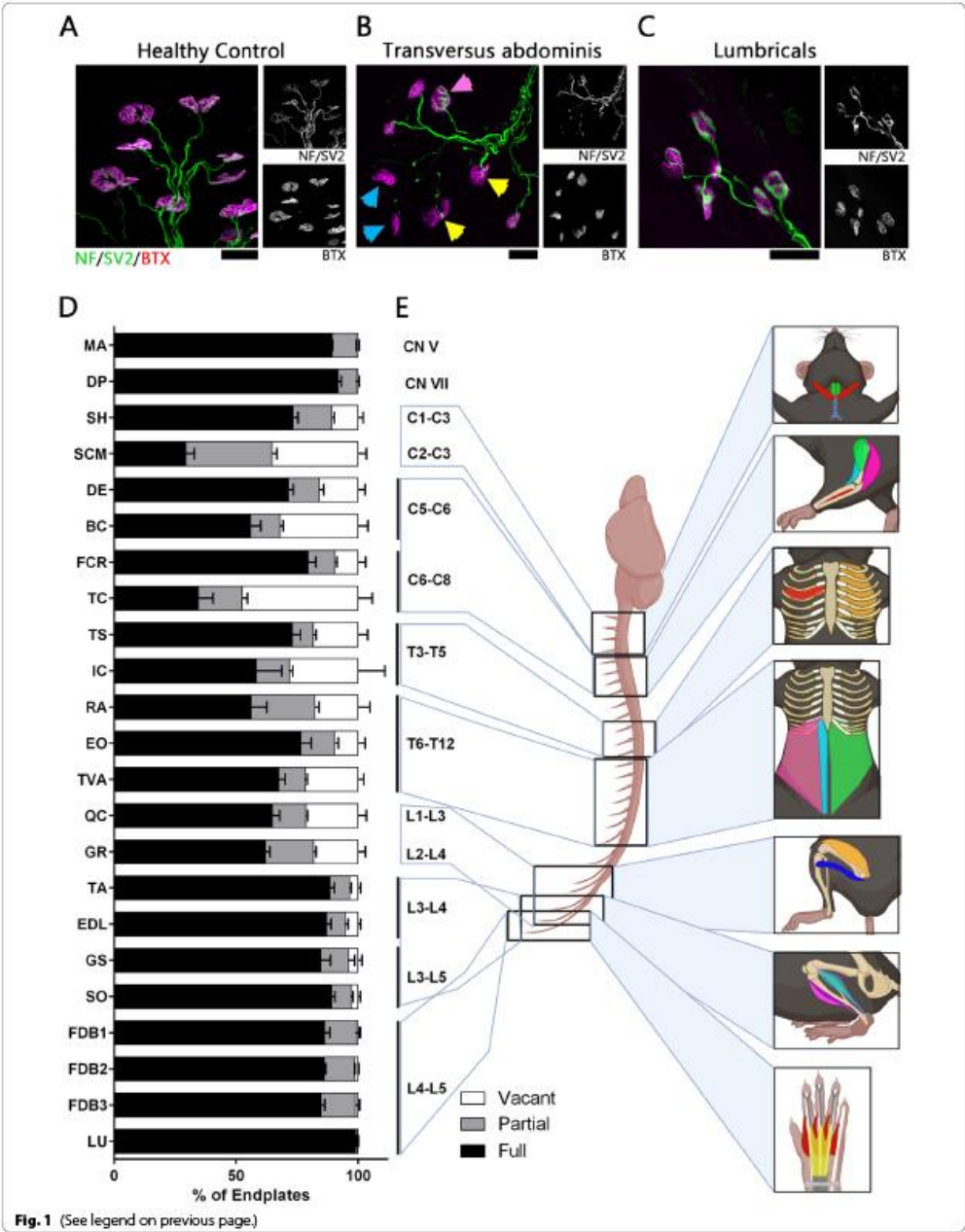


**Fig. 1** (See legend on previous page.)

less fully occupied endplates at the end-stage of the disease. All graphs were created using GraphPad Prism6.

#### RNA-seq data processing and analysis

Three previously published RNA-seq data sets were re-analysed and cross-compared in this study. Raw data for *Smn*<sup>2B/-</sup> mice and their controls were obtained via a kind agreement from the Kothary laboratory [27]), while raw data for *SMNΔ7* mice and their controls was obtained from the Gene Expression Omnibus (Hedlund laboratory, GEO, accession number GSE115706; <https://www.ncbi.nlm.nih.gov/geo/>) and previously published in [41]. Our analysis compared differentially vulnerable motor neurons from cranial nucleus VII in the *Smn*<sup>2B/-</sup> and *SMNΔ7* mouse models of SMA. Moreover, we compared the CNVII derived from both models with *Smn*<sup>2B/-</sup> thoracic spinal cord (tSC) from our previously published data set. Analysis was conducted on data from pre-symptomatic time-points in both models (P2 in *SMNΔ7*; P10 in *Smn*<sup>2B/-</sup>).

The RNA-seq reads from both data sets were mapped to the 10-mm mouse genome assembly. Raw fastq data from the *Smn*<sup>2B/-</sup> data sets were down-sampled to achieve a similar sequencing depth and mapping ratios across all samples of both data sets. Differential gene expression analysis was performed on both data set using DESeq2 package (v. 1.30.1) [47]. Only genes showing a log<sub>2</sub> fold change difference of 1.5 between experimental and control genotype, and with an adjusted *p* value of *p* ≤ 0.05 were considered for the analysis. Lists of differentially expressed genes (DEGs) were produced for both, *Smn*<sup>2B/-</sup> and *SMNΔ7* data sets and DEG overlaps between data sets were obtained. Overlapping DEGs were discarded, and all subsequent analyses were performed on the remaining DEGs of the *Smn*<sup>2B/-</sup> CNVII data set only. Over-representation analysis on GO terms associated with the *Smn*<sup>2B/-</sup>

DEGs was conducted using clusterProfiler (v. 3.18.1). To visualise the expression profile of the GO terms associated genes between samples of both data sets, heatmaps of the *z* scores derived from normalized gene counts were generated. To retrieve networks of protein-protein interaction, we performed a STRING analysis (<https://string-db.org/>, v. 11.5 [48]) on all DEGs from the *Smn*<sup>2B/-</sup> samples (using 433 genes as input), including interactions from database and experimental evidence, with at maximum of 5 interactions for each node of the first shell, and a medium confidence for interaction score to 0.04.

#### Results

##### Higher levels of synaptic loss are present at the centre of the spinal cord in the *Smn*<sup>2B/-</sup> mouse model

Although some differentially vulnerable muscles have been identified in the *Smn*<sup>2B/-</sup> mouse model of SMA [31], a thorough body wide analysis of the muscles was

lacking. Therefore, we performed an extensive analysis of NMJ pathology of 20+ muscles located throughout the body in the *Smn*<sup>2B/-</sup> mouse at the disease end-stage (P16). In our laboratory, this mouse displays a motor phenotype from around P10 and has a life expectancy of around 18 days.

NMJ pathology was analysed by quantification of the percentage of fully innervated, partially innervated, and vacant endplates in immuno-stained muscles from *Smn*<sup>2B/-</sup> and control mice at P16 (Fig. 1A–C, See Supplementary Figure 1 for additional representative images). NMJ pathology was assessed in various muscles from different body regions. The analysis included two head muscles (digastric posterior [DP] and masseter [MA]), two neck muscles (sternocleidomastoid [SCM] and sterno-hyoid [SH]), two thoracic muscles (intercostals [IC] and triangularis sterni [TS]), three abdominal muscles (external oblique [EO], rectus abdominis [RA] and transversus abdominis [TVA]), four forelimb muscles (biceps brachii [BC], deltoid [DE], triceps brachii [TC] and flexor carpi radialis [FCR]), and eight hindlimb muscles (quadriceps [QC], gracilis [GR], extensor digitorum longus [EDL], gastrocnemius [GS], soleus [SO], tibialis anterior [TA], flexor digitorum brevis with its heads on digits 2, 3 and 4 [FDB2, FDB3 and FDB4] and hindlimb lumbricals [LU]). We ordered all investigated muscles by their level of innervation from the spinal cord (Fig. 1D, E). Analysis of the amount of synaptic loss showed that the cranial muscles had lower levels of NMJ loss with 91.8% fully innervated endplates remaining (digastric posterior [DP]). This is consistent with published work from the *Smn*<sup>2B/-</sup>

mouse on other cranial muscles showing no quantifiable denervation at disease end-stage [26, 27]. A higher amount of NMJ loss, with levels of fully innervated endplates as low as 29.3% (sternocleidomastoid [SCM]), could be observed in the neck muscles (SCM and SH) and forelimb muscles (BC, DE, TC and FCR) which are innervated by the cervical spinal nerves. There was also an increased vulnerability within the muscles that are innervated by the thoracic spinal nerves, namely in the thoracic muscles (IC and TS) and the abdominal muscles (EO, RA and TVA). A similar amount of vulnerability with 62% remaining fully innervated endplates (quadriceps [QC]) was also seen in the proximal leg muscles (GR and QC), which are innervated by the upper lumbar spinal nerves. Contrary to the proximal hindlimb muscles, the distal hindlimb muscles (TA, EDL, GS and SO) and foot muscles (FDB2,3,4 and LU), innervated by the middle and lower lumbar spinal nerves, did not show a large amount of synaptic loss. Between 84.8% (gastrocnemius [GS]) and 98.9% (lumbricals [LU]) innervation remained intact in these regions. These observations demonstrate a pattern of vulnerability consistent with increased

susceptibility in the muscles of the core, neck and proximal limbs with lower levels of synaptic loss in the muscles of the distal limbs and face (Fig. 1D, E).

#### Regional NMJ pathology correlates with motor neuron cell body loss in the spinal cord

Data thus far suggests that the muscles innervated by the spinal nerves from the thoracic spinal cord are more vulnerable than the muscles innervated by the lower lumbar spinal nerves. We next investigated whether increased NMJ vulnerability correlated with corresponding motor neuron loss. We have previously shown that motor neuron loss can be detected in the *Smn*<sup>2B/-</sup> mouse model from P15 and is preceded by NMJ loss [15]. We therefore quantified motor neuron somas throughout the ventral horn in T5, T11 and L5 spinal cord segments from P16 mice (Fig. 2). We observed a significant loss of motor neuron somas in T5 and T11 ( $p \leq 0.05$ ). There was no significant difference in the total number of motor neurons at L5. These results indicate that NMJ loss is associated with the loss of motor neuron somas and provides further evidence that there is an increased vulnerability of motor neurons in the thoracic region compared to lower lumbar levels.

#### Distinct patterns of selective vulnerability

##### In the *Smn*<sup>-/-</sup>; *SMN2*, Taiwanese, *SMNΔ7* and *Smn*<sup>2B/-</sup> mouse models

Previous studies have utilised a range of available mouse models to classify the relative vulnerability of the muscles in SMA mouse models [14, 20, 24–29]. In order to gain insight into consistency of susceptibility patterns across SMA mouse models, we compared patterns of selectively vulnerable muscles across the four most commonly used SMA mouse models: the *Smn*<sup>-/-</sup>; *SMN2*, Taiwanese, *SMNΔ7* and *Smn*<sup>2B/-</sup> mouse model, supplemented by our own analysis from the *Smn*<sup>2B/-</sup> mouse model (Fig. 1) and *SMNΔ7* (Supplementary Figure 2) [14, 20, 24, 25, 27–29]. Towards this goal, we developed a seven-point colour-coded vulnerability classification tool. The muscles were classified based upon the percentage of fully innervated motor endplates, and each category was given a specific colour from dark green to dark magenta to represent a

10% range of fully occupied endplates (see legend in Fig. 4).

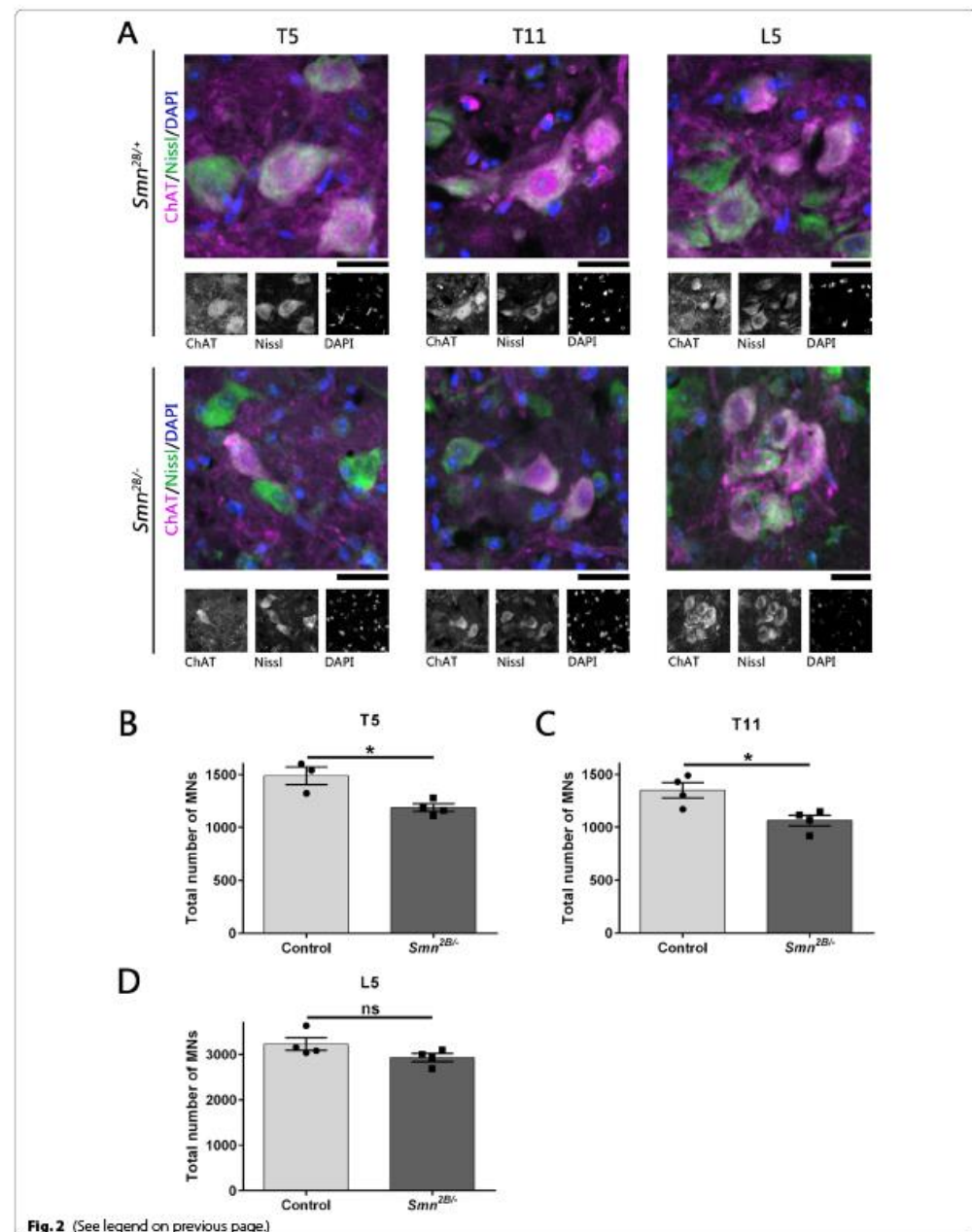
We found that in mouse models, the muscles appear to sit on a spectrum, displaying many different levels of vulnerability, rather than showing a bimodal split in relative vulnerability (Fig. 3). Furthermore, there appears to be a similar range in relative vulnerability across most models. In the *Smn*<sup>-/-</sup>; *SMN2* mouse model, vulnerability levels range from >99% of endplates remaining fully occupied in the adductor auris longus [14] to 36% remaining fully occupied endplates in the caudal band of the levator auris longus [25] (Fig. 3A). The percentage of innervated endplates in the *SMNΔ7* mouse models ranged from 100% in the quadriceps [20] to 15% in the auricularis superior (Fig. 3C, Supplementary Figure 2). In the *Smn*<sup>2B/-</sup> mouse model, innervation ranged from 99% in the lumbrical muscles to 30% in the sternocleidomastoid (Fig. 3D). In the Taiwanese mouse model, the variability in vulnerability again followed a similar range (98% innervation in the semispinalis capitis to 23% innervation in the flexor digitorum brevis 2) [29]; Fig. 3B). However, it is notable that the majority of the muscles are comparatively resistant to synaptic loss in this model. These findings show that all four mouse models display a similar range of NMJ loss, but that the Taiwanese model shows a milder degree of NMJ pathology compared to the other three mouse models.

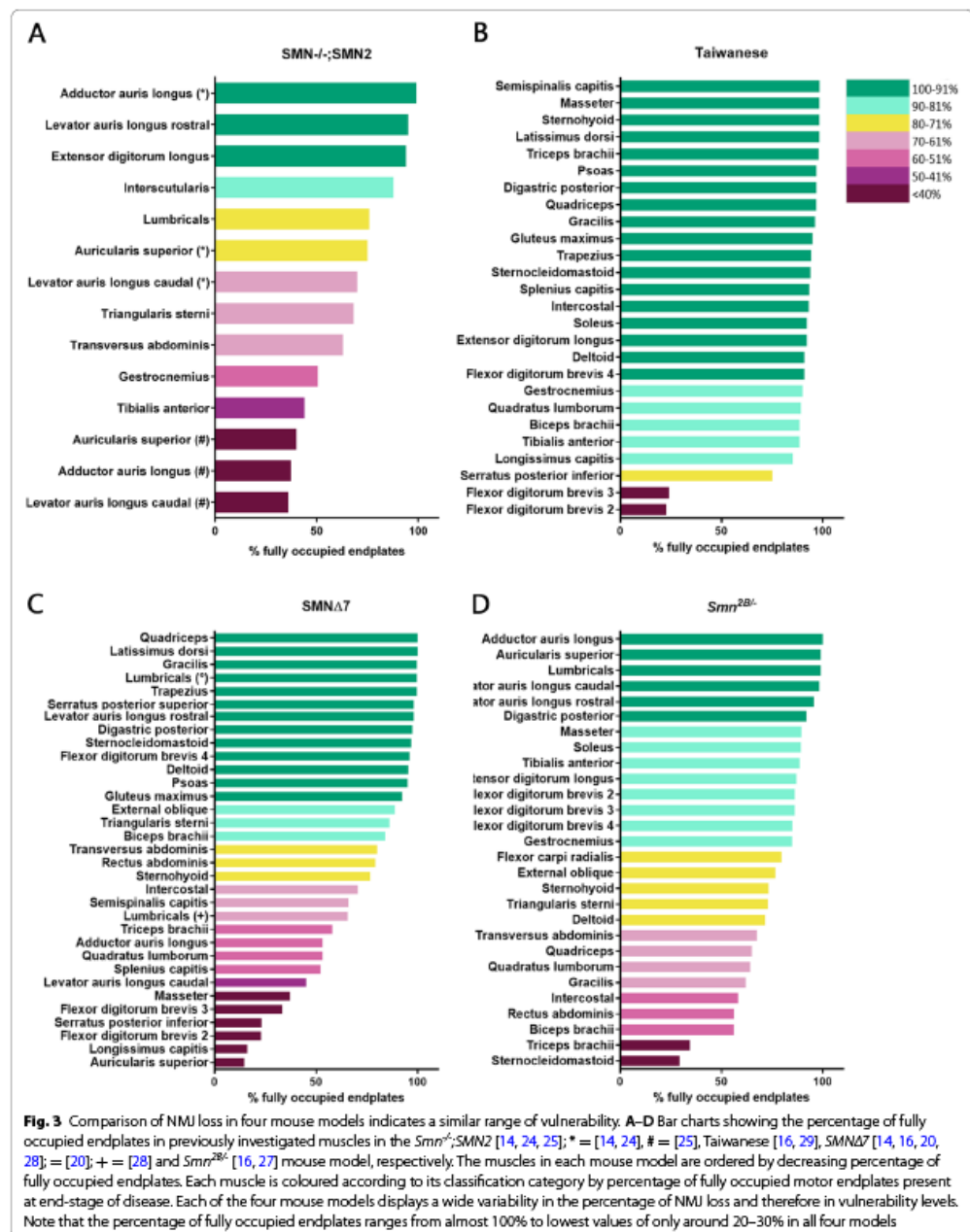
To gain a better understanding of the similarities and differences in vulnerability patterns of the mouse models, we used our analysis and available published data to compare specific muscles (Fig. 4). The table displays all previously investigated muscles in the *Smn*<sup>-/-</sup>; *SMN2*, Taiwanese, *SMNΔ7* and *Smn*<sup>2B/-</sup> mouse models, categorised by anatomical body regions. Each muscle in the table is displayed in the colour of the allocated category of our classification tool, based on its number of remaining fully occupied endplates (Fig. 4).

Analysis of this data by body region revealed that the vulnerability of a given muscle varied considerably between mouse models. Firstly, the regional pattern of vulnerability wherein there was an increase in synapse loss in core and proximal limb muscles which was evident in the *Smn*<sup>2B/-</sup> mouse, was not seen in any of the other three models. Indeed, in the Taiwanese mouse

(See figure on next page.)

**Fig. 2** Significant motor neurons loss is observed in thoracic spinal cord segments in the *Smn*<sup>2B/-</sup> mouse model **A** Representative images showing motor neurons (MNs) labelled with an antibody against choline acetyltransferase (ChAT; magenta), as well as Nissl (green) and DAPI (blue) staining in *Smn*<sup>2B/-</sup> and *Smn*<sup>2B/+</sup> control mice in thoracic spinal cord segments (T5 and T11) and lumbar spinal cord segment L5 at P16. Scale bar: 50μm. **B–D** Bar charts showing the total number of MNs quantified in the ventral horn in the spinal cord segments T5, T11 and L5 in *Smn*<sup>2B/-</sup> mice compared to *Smn*<sup>2B/+</sup> controls. There was a significant decrease in the total number of MNs in *Smn*<sup>2B/-</sup> mice compared to control in both thoracic spinal cord segments. However, there was no significant difference in the total number of MNs in the lumbar segment. Unpaired Student's *t* test (ns = no significance, \* $p \leq 0.05$ ).  $n=3,4$  for controls and *Smn*<sup>2B/-</sup> mice respectively in T5;  $n=4$  for both genotypes in all other spinal cord segments. Error bars represent mean  $\pm$  SEM





model, the majority of muscles display between 100 and 81% fully innervated endplates. The only exceptions with severe NMJ loss were the FDB 2 and 3 of the foot muscles. In the *Smn*<sup>-/-</sup>; *SMN2* and *SMNΔ7* mouse models, many body regions include both vulnerable and resistant muscles. In the *SMNΔ7* mouse model, the frequency of this dichotomy of vulnerability in certain body regions is particularly prominent and especially pronounced in the neck muscles. Three muscles are extremely vulnerable and two are moderately affected, whilst two are largely unaffected by pathology. In order to confirm that the variability in relative vulnerability was not due to observer effects, we quantified a range of muscles in the *SMNΔ7* and *Smn*<sup>2B/-</sup> mice in our laboratory (Supplementary Figure 3). This verified the occurrence of differing levels of pathology between mouse models in the muscles such as the auricularis auris longus, auricularis superior, triangularis sterni, external oblique and rectus abdominis.

These profound differences between models can best be highlighted when looking at some examples of how individual muscles differ across models. For example, AS, AAL and LALC, three cranial muscles, are severely affected in the *SMNΔ7* model and the *Smn*<sup>-/-</sup>; *SMN2* mouse models, but resistant in the *Smn*<sup>2B/-</sup> mouse model. In the Taiwanese model, almost all innervation is remaining in the TC muscle, while in the *SMNΔ7* model, 57.9% endplates remain fully innervated, and only 34.4% endplates remain occupied in the *Smn*<sup>2B/-</sup> mouse model. The TA muscle is >90% fully occupied in the *SMNΔ7* and *Smn*<sup>2B/-</sup> mouse models, but 44% fully occupied in the *Smn*<sup>-/-</sup>; *SMN2* mouse model. The IC muscles also behave differently across all models, where there is around 55% and 70% of NMJ remaining innervated in the *Smn*<sup>2B/-</sup> model and *SMNΔ7* mice, respectively, and almost no synaptic loss in the Taiwanese model.

In order to explore other factors which may affect relative vulnerability, the muscles were grouped based upon their fast synapsing (FaSyn) and delayed synapsing (DeSyn) phenotype, or muscle fibre type (Supplementary Figure 4). This revealed no correlation in any of the four models analysed.

These findings show that comparison of selective vulnerability patterns revealed four distinct patterns of selective vulnerability in commonly used mouse models of SMA. This discovery of profound differences in vulnerability patterns between mouse models is very important to consider during experimental design. It can also be a tool to better understand the mechanisms driving selective vulnerability in different mouse models of SMA. Further work is needed to uncover the mechanisms underlying NMJ and motor neuron vulnerability in these mouse models of disease.

#### Transcriptional analysis on differentially vulnerable motor neurons reveals groups of transcripts that could contribute to motor neuron protection

To investigate the molecular differences which may underlie the contrasting patterns of selective vulnerability, we conducted a cross-model transcriptional analysis of differentially vulnerable motor neurons. We focused on facial motor neurons as their innervation remains largely intact in the *Smn*<sup>2B/-</sup> model, whereas in the *SMNΔ7* mouse model, >40% of the innervation is lost (Fig. 4). We therefore reasoned that unique transcriptional changes occurring in the facial motor neurons of the *Smn*<sup>2B/-</sup> mouse may confer protection.

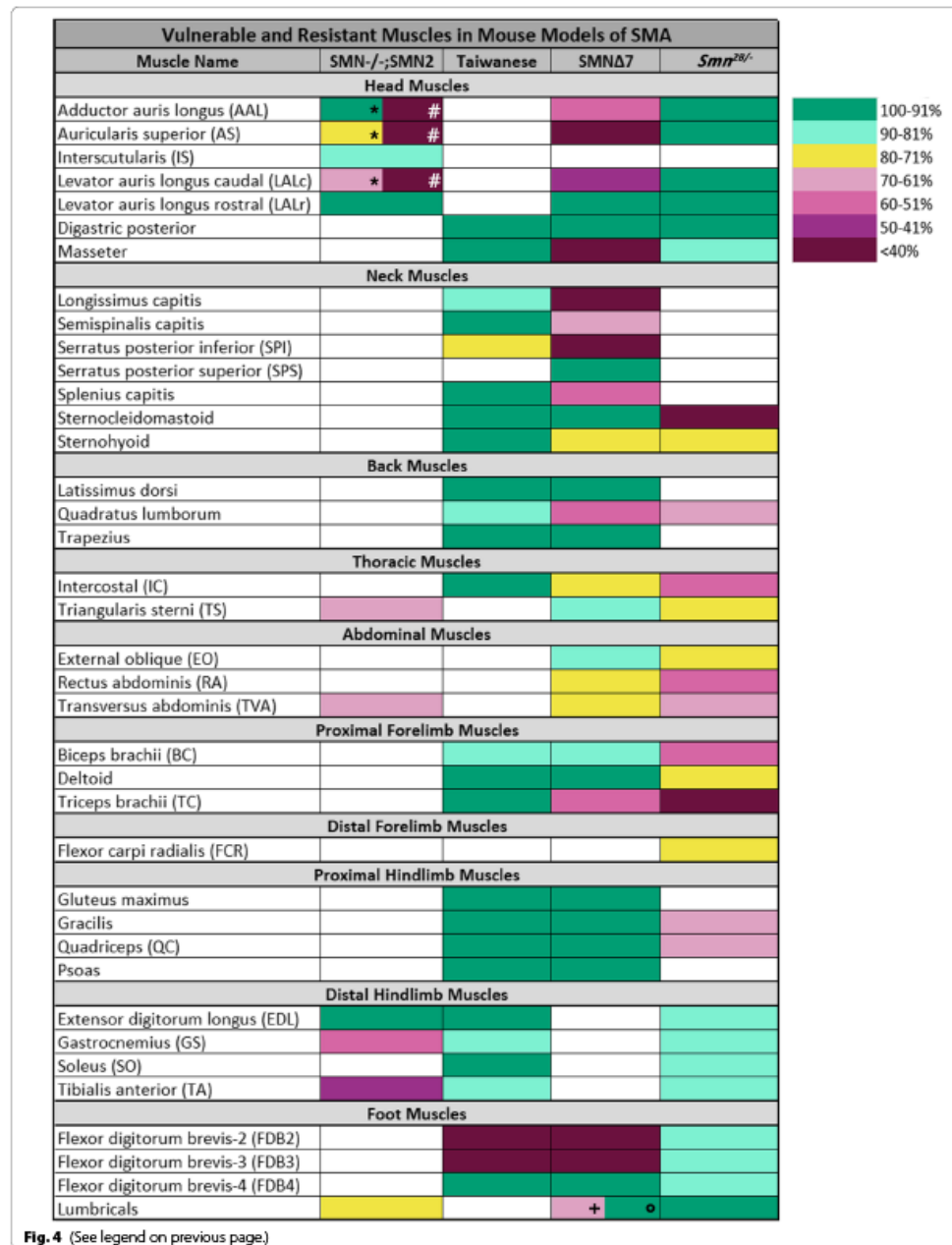
In previous studies, we have individually performed transcriptional analysis on facial motor neurons from *Smn*<sup>2B/-</sup> mice at P10 or *SMNΔ7* mice at P2, P5 and P10 compared to their respective controls [27, 41]. Here, we cross-compared the data sets at a comparable pre-symptomatic time-point (P10 in the *Smn*<sup>2B/-</sup> and P2 in the *SMNΔ7* mouse model) and compiled a list of DEGs in the resistant CNVII motor neurons in the *Smn*<sup>2B/-</sup> model (Fig. 5A).

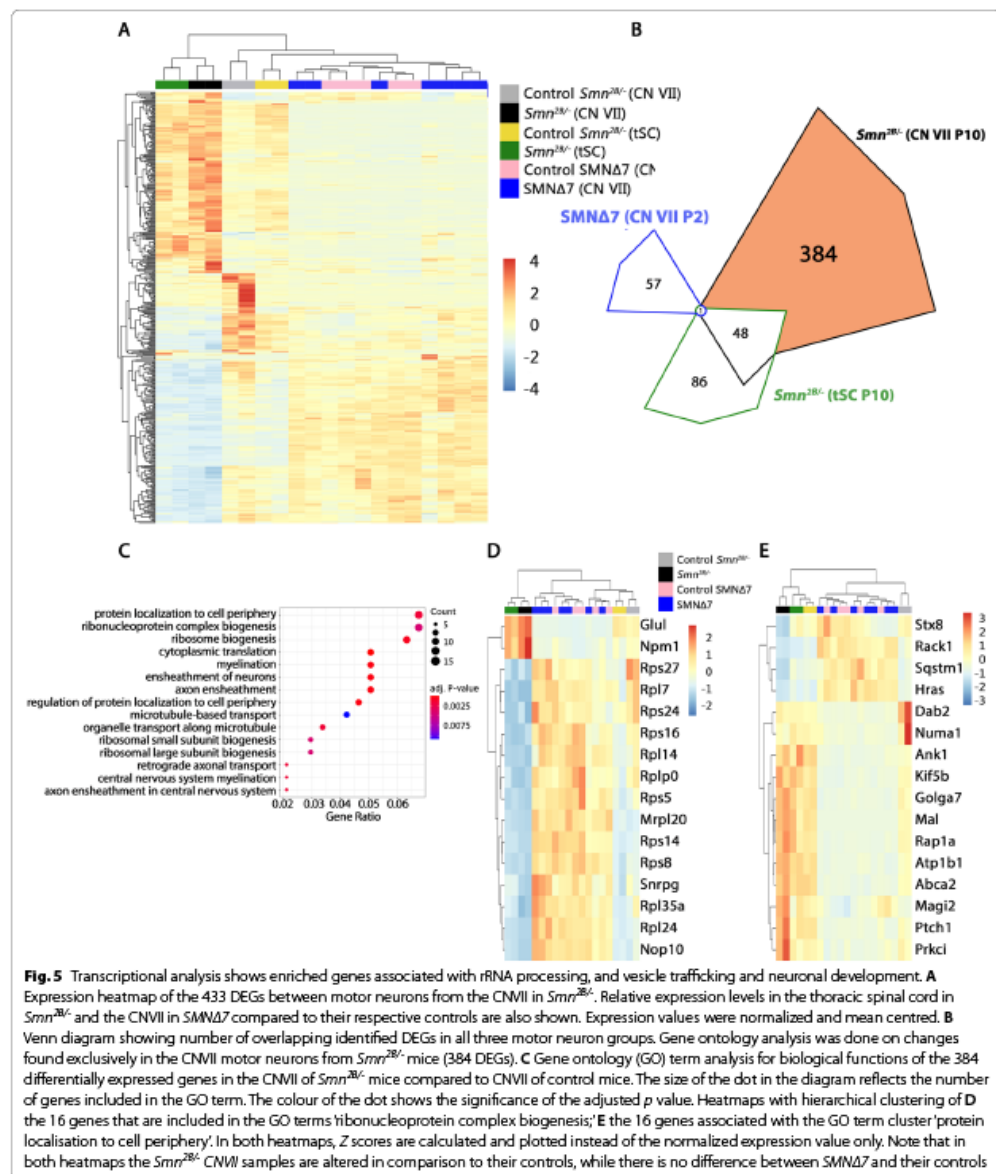
Hierarchical clustering using the DEGs of the resistant CNVII in the *Smn*<sup>2B/-</sup> model showed a clear separation of the altered DEGs between this model and the *SMNΔ7* model (Fig. 5A).

We then compiled a group of 384 DEGs which were unique to the *Smn*<sup>2B/-</sup> CNVII data set, by removing any transcripts which were also altered in *Smn*<sup>2B/-</sup> vulnerable

(See figure on next page.)

**Fig. 4** Comparison of NMJ loss in four mouse models indicates diverse patterns of selective vulnerability. The colour gradient legend describes seven classification categories of the NMJ pathology according to the percentage of fully occupied motor endplates in a muscle at disease end-stage (see 'Methods' section for more details). The table shows investigated muscles coloured according to their classification categories by percentage of fully occupied endplates in the *Smn*<sup>-/-</sup>; *SMN2* [14, 24, 25], Taiwanese [16, 29], *SMNΔ7* [14, 16, 20, 28] and *Smn*<sup>2B/-</sup> [16, 27] mouse model. The table displays the muscles categorised by body regions (head, neck, back, etc.). Note that for some muscles, different results were observed in two independent studies. This occurred in the AAL, AS and LALC muscles \* = [14, 24]; # = [25] in the *Smn*<sup>-/-</sup>; *SMN2* model and the LU + = [28] = [20] in the *SMNΔ7* model. In the *Smn*<sup>-/-</sup>; *SMN2* mouse model, all body regions seem to be moderately affected and display different vulnerability levels, with the most severely affected the muscles in the hindlimb. The Taiwanese mouse model shows very little vulnerability, except for the FDB 2 and FDB 3. The vulnerability pattern in the *SMNΔ7* mouse model shows that the head, neck and foot muscles are the most affected, followed by ventral core muscles (thoracoabdominal muscles) and the least vulnerability is seen in the limb and back muscles. The *Smn*<sup>2B/-</sup> mouse model displays a regional vulnerability pattern, where the neck muscles, core muscles (thoracoabdominal muscles) and proximal fore- and hindlimbs seem more affected than the head muscles, distal limb and foot muscles





tSC motor neurons (48 transcripts) or *SMNΔ7* CNVII motor neurons (1 transcript) compared to their respective controls (Fig. 5B). To interpret the biological

processes dysregulated in *Smn*<sup>2B/-</sup> facial motor neurons, we used the 384 DEGs unique to these and conducted a Gene Ontology (GO) term enrichment analysis (Fig. 5A).

We identified a number of GO terms, including 'protein localisation to cell periphery' [GO:1904375] and 'ribonucleoprotein complex biogenesis' [GO:0022613] (Fig. 5D, E). The 'ribonucleoprotein complex biogenesis' GO term cluster included 16 transcripts and had an adjusted  $p$  value of  $p=0.004$ . Here, genes were associated with cell signalling, rRNA processing and maturation, and ribosome constituents and biogenesis (Fig. 5D). The GO term cluster 'protein localisation to cell periphery' included 16 differentially expressed transcripts and had an adjusted  $p$  value of  $p=0.002$ . Genes in this cluster were associated with vesicle trafficking, microtubule dynamics and neuronal development (Fig. 5E).

In order to visualise the interaction between these networks and other dysregulated transcripts, we used the STRING database to retrieve networks of protein-protein interactions from all DEGs of *Smn*<sup>2B/-</sup> CNVIII samples at P10 (Fig. 6). Ribosomal proteins from the small subunit (*Rps* proteins) and large subunit (*Rpl* proteins) formed a dense network, consistent with the GO term analysis. Most of the genes in this network contribute to ribosome biogenesis, including *Rps5*, *Rps16*, *Rpl24* and *Rps14*. Other genes like *Rps27*, *Rps24* and *Snrpg* contribute to pre-rRNA and rRNA processing and maturation. Among the DEGs with a role in vesicle trafficking, we found genes that are important for neuronal development and neurite outgrowth (*Magi2*, *Kif5b* and *Rap1a*), and microtubule dynamics (*Numa1*, *Kif5b* and *Prkci*). Furthermore, the network also included *Sqstm1*, which is connected to frontotemporal dementia and amyotrophic lateral sclerosis. Lastly, *Snrpg*, which was included in the GO term analysis, plays a role in RNA splicing as component of the SMN spliceosome assembly. In summary, we identified several transcriptional programs that are specifically activated in the facial motor neurons from *Smn*<sup>2B/-</sup> animals and which may contribute to the protection of motor neurons in this model.

## Discussion

The findings in this study provide novel insights into the selective vulnerability patterns of mouse models of SMA and reveal groups of transcripts that could contribute to motor neuron protection. In this study, we have conducted a thorough body wide analysis of the selective vulnerability pattern present in the *Smn*<sup>2B/-</sup> mouse model at the disease end-stage and show that the *Smn*<sup>2B/-</sup> model displays a regional pattern of NMJ loss. Comparison of vulnerability patterns in four SMA mouse models reveals dramatic differences in patterns of selective vulnerability between models. Finally, we show that an increase in transcripts associated with ribosomal bioenergetics, vesicle trafficking and neuronal development correlates with protection of motor neurons from degeneration.

## Contributing factors of differential vulnerability in the four most commonly used mouse models of SMA

Neuromuscular pathology within the *Smn*<sup>2B/-</sup> mouse model displays a unique regional vulnerability pattern. This regional vulnerability pattern is a feature that was previously documented in SMA patients, where patients present with an increased vulnerability and earlier weakness in the core muscles, proximal leg and proximal arm muscles [21–23]. There is a common sparing of the muscles innervated by the facial nerve and distal lower limbs. We also observed an increased vulnerability of the proximal limbs compared to the distal limbs, and increased vulnerability of the core and neck muscles which has been observed in patients [21, 22]. A comprehensive study profiling patterns of vulnerability in patients will become increasingly pertinent, especially if we see the predicted correlation between relative vulnerability and treatment efficacy [34]. Elucidating patterns of selective vulnerability from autopsy material is not optimal, since the temporal order of motor neuron loss cannot be discerned. Due to clinical intervention and care, human autopsy material tends to reflect a later disease stage than mice at 'end-stage', which may be one reason why motor neuron loss in patients appears more profound than in mice. Regardless, based on the currently available data, the correlation between the regional patterns of vulnerability in SMA patients and the *Smn*<sup>2B/-</sup> mouse model are intriguing and may support the use of this mouse model in the identification of protective disease modifiers of potential clinical relevance.

The analysis of synaptic loss in the *Smn*<sup>2B/-</sup> mouse model presented here shows that the muscles innervated by cervical, thoracic and upper lumbar spinal nerves are more vulnerable than the muscles innervated by the cranial nerves and lower lumbar spinal nerves. The reasons for the selective vulnerability of this body region are unclear. Whilst motor unit type has been shown to be a key regulator of selective vulnerability in ALS [49, 50], there is no correlation between fibre type and relative vulnerability here, which is consistent with previous work in the *SMNΔ7* mouse model [20]. Indeed, muscle fibre type does not correlate with relative vulnerability in any of the four mouse models examined. Further, it was suggested that the subdivision of NMJs with respect to their development and stability is a contributing factor to selective vulnerability [14, 51]. The muscles can be subdivided into fast synapsing (FaSyn) and delayed synapsing (DeSyn) muscles according to their intrinsically distinct features of the rate at which they acquire their organisation of mature NMJs [51]. The FaSyn muscles were found to be more vulnerable in the head muscles of an SMA mouse model than in the DeSyn muscles [14]. Our study rules out muscle fibre type or synapsing rate as key factors for motor

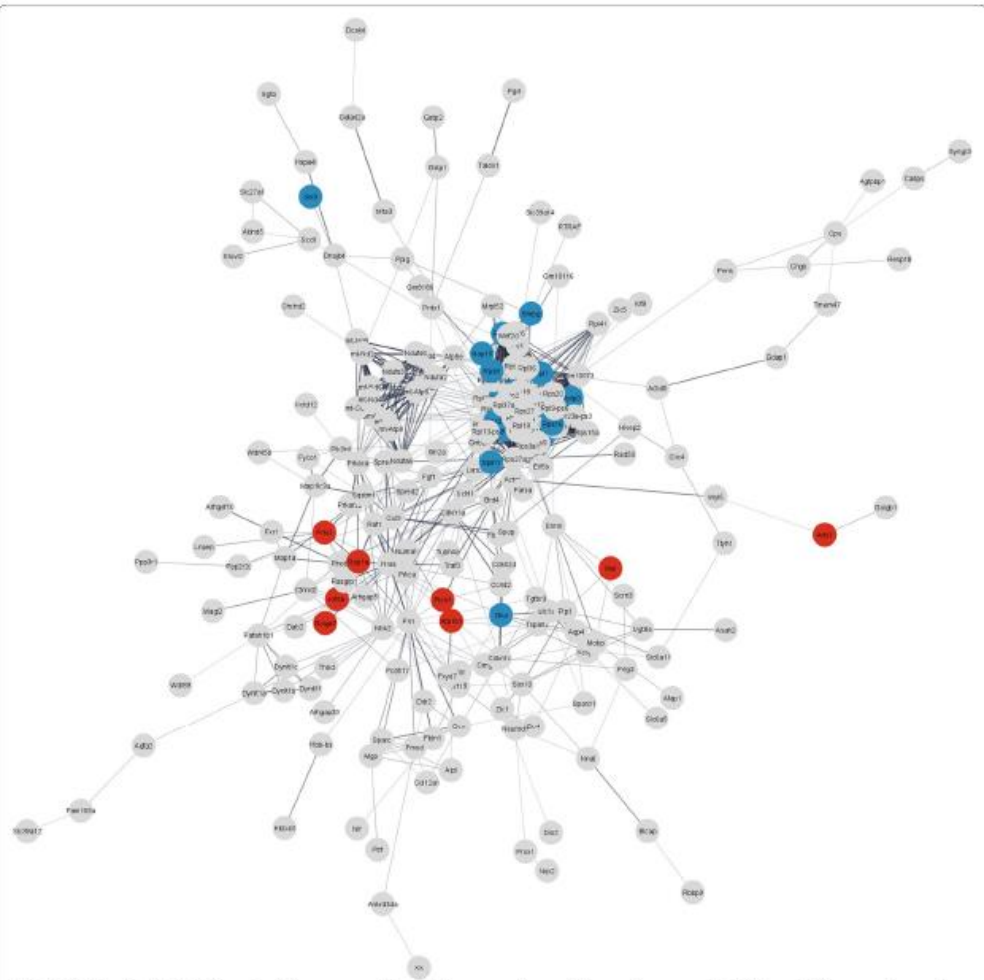


**Fig. 6** STRING analysis of DEGs from *Smr*<sup>2B/-</sup> mouse model reveals large protein-protein interaction network. STRING analysis for protein-protein interactions including all differentially expressed genes regulated in the CNVII of the *Smr*<sup>2B/-</sup> mouse model of SMA compared to control littermates. Nodes are coloured according to selected GO terms: (red) protein localisation to cell periphery and (blue) ribonucleoprotein complex biogenesis. Isolated nodes were removed. Network edges represent the confidence of the predicted associations between nodes (edge thickness = strength of data support)

neuron vulnerability. This is in line with previously published data from the *SMNΔ7* mouse model, where some FaSyn muscles were also identified as resistant to pathology [20]. Lastly, it has been proposed that the location of motor neurons within the spinal cord could be responsible for determining their vulnerability in SMA, whereby

those located medially, innervating axial and proximal limb muscles are more vulnerable than those located laterally, innervating the distal limb [52, 53]. In support of this, in the *SMNΔ7* model, there was a higher degree of sensory motor breakdown at L1 (innervating proximal leg) than at L5 (distal leg) [52]. Analysis of motor neuron

size in the *SMNΔ7* mouse also revealed a selective shrinkage of motor neurons innervating the proximal forelimb, compared to distal forelimb and phrenic motor neurons [53]. The current study shows that a similar pattern is present in the *Smn*<sup>2B/-</sup> model, where vulnerability of muscles is most pronounced in the axial, proximal forelimb and proximal hindlimb muscles. Although this pattern does not bear out in the other mouse models of SMA, there appears to be an intriguing correlation between muscle location and relative vulnerability in at least the *Smn*<sup>2B/-</sup> mouse model of SMA and in patients with SMA. It is often assumed that proximal motor units are larger than those found most distally. It is therefore tempting to speculate that increased motor unit size drives vulnerability. This idea has been previously discounted during a study on selective vulnerability in the *Smn*<sup>-/-</sup>;*SMN2* mouse model [25]. However, since the correlation between motor unit size and relative vulnerability was only performed on a small number of the muscles, all with comparatively small motor units, and did not include any axial or proximal limb muscles, this idea should be revisited.

#### **Dramatic difference in vulnerability patterns between mouse models of SMA**

We compared the NMJ pathology of the four most commonly used mouse models of SMA and showed that each model displays a distinct pattern of selective vulnerability. This is consistent with a previous report where NMJ pathology in the tibialis anterior (TA) muscle and quadratus lumborum (QL) muscles were compared in three mouse models [16]. Whilst TA was consistently less vulnerable, levels of denervation in the QL varied between models from 0 to 55%. All of the investigated mouse models share a common mutation within the *Smn* gene to mimic the phenotype of human disease [31, 54–56]. Despite their shared mutation in the same gene, there is a drastic difference between the observed vulnerability patterns of the muscles between models and the reasons for this remain unclear. Comparing these different mouse models of SMA and their divergent patterns of selective vulnerability therefore creates a valuable opportunity to further understand the mechanisms which drive motor neuron pathology in SMA. Whilst the mouse strains do vary between these models, it cannot explain the full extent of the disparities. Indeed, the Taiwanese model is on the same background as the *Smn*<sup>-/-</sup>;*SMN2* and *SMNΔ7* models, yet their pattern of NMJ loss is vastly different. This is consistent with previous reports where differences in mouse strain did not account for the variation in the severity of phenotype or degree of motor neuron loss observed between mouse models [16]. One possible explanation for this is relative *Smn* levels, which are higher in the Taiwanese compared to the *SMNΔ7* model.

However, since *Smn* levels are similar between *Smn*<sup>2B/-</sup> and Taiwanese mice, this cannot fully account for the disparities and further work will be required to discern why motor neuron pathology is less severe in this mouse model.

Previous work has identified important differences between mouse models of SMA. Motor neuron loss appears comparatively milder in the Taiwanese mouse model and this is consistent with our findings here (Figs. 3 and 4) [16, 25]. Other disparities between models have been previously noted, included selectively vulnerable regions of the spinal cord and P53 pathway activation [16]. This current work supports these important differences between models and highlight the need to consider the impact of the chosen model and experimental design on conclusions.

On possible explanation to account for the different patterns of vulnerability in the *Smn*<sup>2B/-</sup> mouse compared to other models is postnatal age. Previous work has shown that postnatal age can influence the pathways responsible for axon degeneration and that different muscles mature at different rates [44, 45, 57]. During the first postnatal month, the neuromuscular system undergoes dynamic molecular and structural changes associated with postnatal maturation, including developmental synapse elimination and consolidation and maturation of remaining connections. During the time period, the rate of synaptic degeneration following traumatic or hypoxic injury accelerates [44, 45]. The acceleration in degeneration following injury has been attributed to postnatal changes in mitochondria, which are progressively enriched in both muscle and peripheral axons during the first postnatal month [57, 58]. Interestingly, bioenergetic status has also been proposed as a regulator of selective vulnerability in mouse models of SMA [40]. Developmental regulation of neuronal tissue dynamics could play an important role in the development of different vulnerability pattern. If we consider these multiple developmental process regulations, coupled with the fact that all models have a different age of onset, they pose a possible hypothesis as to why the patterns of selective vulnerability vary so drastically between models. Investigating how mitochondria change in the synapses, axons and cell bodies in the different motor units from different SMA mouse models could potentially shed light on the reasons for the differences in patterns of vulnerability between mouse models of SMA.

#### **Potentially important molecular pathways for motor neuron protection**

By conducting a cross-model transcriptional analysis of motor neuron vulnerability, we show that certain groups of transcripts are candidate protective modifiers. Our

analysis revealed an enrichment in transcripts associated with the GO term 'ribonucleoprotein complex biogenesis'. Transcripts in this cluster contribute to ribosome biogenesis as part of the ribosomal proteins from the small and large subunit, as well as to pre-RNA and rRNA processing and maturation, and regulation of the translation apparatus. Since SMN and associated proteins have been implicated in both pre-mRNA splicing and in the regulation of translation [59–62], it is tempting to speculate that a change in the regulation of these pathways could account for why this subset of neurons are more resistant to a reduction in SMN levels. To further investigate this aspect, transcriptomics should be coupled with proteomics to delineate modifications in proteostasis that may counteract detrimental effects of SMN loss.

We also demonstrated an enrichment in the transcripts associated with the GO term 'protein localisation to cell periphery'. Transcripts in this cluster contribute to cellular pathways concerned with vesicle trafficking, microtubule dynamics and neuronal development. *Rap1a*, *Prkci*, *Magi2*, *Hras*, and *Numa1* are all part of the *Ras* and *Rap1* signalling pathways. The *Ras* signalling pathway transduces signals from the extracellular milieu to the cell nucleus, activating specific genes for cell growth, division and differentiation. The *Rap1* signalling pathway controls cellular processes, such as cell migration, cell proliferation and cell survival. *Magi2*, *Kif5b*, *Sqstm1* and *Rap1a* are associated neuronal development and neurite outgrowth, and some have previously been implicated in the pathogenesis of neurodegenerative diseases. For example, *Kif5b* is an axonal kinesin involved in anterograde axonal transport. It is part of signalling pathways in various neurodegenerative diseases including Huntington's disease, Parkinson's disease and ALS. Another member of the kinesin family (*Kif5a*) contributes to cytoskeletal defects in the pathogenesis of ALS [63]. It has also been demonstrated that gene mutations associated with an ALS mouse model interfere with kinesin members and facilitate defects in axonal transport that ultimately lead to death of motor neurons [64]. We suggest that *Kif5b* and other kinesin members have the potential to also contribute to the pathogenesis of other motor neuron diseases like SMA. Furthermore, several of these transcripts, as mentioned, are important for axon function. Since SMN has pivotal functions in the axon and SMA is an axonopathy, several of these genes could be regulated as compensation for the loss of SMN to maintain axon function [65–69].

Another transcript in the enriched clusters of our analysis is *Sqstm1*. *Sqstm1* is further associated with mitophagy, the removal of damaged mitochondria through autophagy processes. The *Sqstm1* gene encodes p62, and mutations in the *Sqstm1* gene have previously

been reported in both ALS and the related disorder frontotemporal dementia. The deficiency of p62 was associated with inhibited complex I mitochondrial respiration, due to deficits in the electron transport chain [70]. Silencing or knockout of p62 in animal models resulted in mitochondrial dysfunction [71–73]. These findings taken together demonstrate that dysfunction of the *Sqstm1* gene is strongly associated with pathophysiological events in energy metabolism. Since bioenergetics status was previously found to be implicated in motor neuron vulnerability [40], we suggest that upregulation of *Sqstm1* could be protective modifier of motor neuron pathology in SMA. Increasing the expression of *Sqstm1* and other potential modifiers identified here in vivo will give further insight about their neuroprotective properties.

## Conclusions

In summary, the data presented above represents a detailed analysis of selective vulnerability pattern in four mouse models of SMA. Selective vulnerability patterns differ across all 4 mouse models, with the regional pattern in the *Smn*<sup>2B/-</sup> mouse showing similarity to the regional pattern in SMA patients. Based on these findings, we identified populations of motor neurons that are differentially vulnerable in the *Smn*<sup>2B/-</sup> and *SMNΔ7* mouse model of SMA. We show that transcripts associated with cellular functions which are known to be perturbed in deficient motor neurons, such as RNA processing, axonal transport and bioenergetics status, are differentially regulated in selectively resistant neurons. These novel insights reveal promising targets that will be interesting to further investigate in the context of motor neuron protection in spinal muscular atrophy.

## Abbreviations

ALS: Amyotrophic lateral sclerosis; AAL: Adductor auris longus; AS: Auricularis superior; BC: Biceps brachii; BTX: α-Bungarotoxin; CN VII: Cranial nerve nucleus VII; DE: Deltoid; DeSyn: Delayed synapsing; DEG: Differentially expressed gene; DP: Digastric posterior; EDL: Extensor digitorum longus; EO: External oblique; FaSyn: Fast synapsing; FCR: Flexor carpi radialis; FD8: Flexor digitorum brevis; GO: Gene Ontology; GR: Gracilis; GS: Gastrocnemius; IC: Intercostals; LALC: Levator auris longus caudal; LALR: Levator auris longus rostral; LU: Lumbricals; MA: Masseter; MN: Motor neuron; NF: Neurofilament; NMJ: Neuromuscular junction; QC: Quadriceps; RA: Rectus abdominis; SCM: Sternocleidomastoid; SH: Sternohyoid; SMA: Spinal muscular atrophy; SMN: Survival motor neuron protein; *SMN1*: Survival motor neuron 1 gene; SO: Soleus; TA: Tibialis anterior; TC: Triceps brachii; TS: Triangularis sterni; TVA: Transversus abdominis.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13395-022-00305-9>.

**Additional file 1: Supplementary Figure 1.** Representative images from *Smn*<sup>2B/-</sup> mice. Confocal images from muscles showing NMJs labelled with antibodies against NF (green), SV2 (green) and BTX (red) from control (*Smn*<sup>2B/+</sup>) and *Smn*<sup>2B/-</sup> mice from muscles located in the head and neck

(A,C), thoracoabdominal region (D,F), forelimb (G,I) and hind limb (J,N). Schematic images (B, E, H, K–M) show location of muscles, with colour linking to confocal images from specific muscles. Scale bar = 25  $\mu$ m.

**Additional file 2: Supplementary Figure 2.** Analysis of synaptic loss in cranial and thoracoabdominal muscles in SMN $\Delta$ 7 mouse model at P12. (A) Schematic diagram of the anatomical locations of cranial muscles (adductor auris longus [AAL] and auricularis superior [AS]) in a dorsally viewed mouse. (B) Schematic diagram of the anatomical locations of thoracic muscles (triangularis sterni [TS]) in a mouse in supine position. (C) Schematic diagram of the anatomical locations of abdominal muscles (external oblique [EO] and rectus abdominis [RA]) in a mouse in supine position. (D–F) Bar charts showing the quantification of percentage of full, partial and vacant endplates in SMN $\Delta$ 7 mice compared to controls in cranial muscles (AAL and AS), thoracic muscles (TS), and abdominal muscles (EO and RA) respectively. Note that all cranial muscles show a significant increase in vacant motor endplates and a significant decrease in fully occupied endplates, whereas non of the thoracic or abdominal muscles show a significant increase in vacant endplates. Two-sided ANOVA with Sidak correction (ns = no significance, \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$  and \*\*\*\* $p \leq 0.0001$ ).  $n=3$  for controls and SMN $\Delta$ 7 mice respectively. Error bars represent mean  $\pm$  SEM.

**Additional file 3: Supplementary Figure 3.** Comparison of levels of synaptic loss between the SMN $\Delta$ 7 mouse model and the Smn2B/- mouse model in muscles of three different body regions. (A–C) Bar charts showing the quantification of percentage of full, partial and vacant endplates in SMN $\Delta$ 7 mice compared to Smn2B/- mice in cranial muscles (adductor auris longus and auricularis superior), thoracic muscles (triangularis sterni), and abdominal muscles (external oblique and rectus abdominis) respectively. Note that all SMN $\Delta$ 7 cranial muscles show a significant increase in vacant motor endplates and a significant decrease in fully occupied endplates compared to the Smn2B/- cranial muscles, whereas Smn2B/- thoracic and abdominal muscles are significantly more vulnerable than in SMN $\Delta$ 7 mice. Significance levels indicate the statistical difference between full, partial and vacant endplates when comparing SMN $\Delta$ 7 to Smn2B/- mice. Two-sided ANOVA with Sidak correction (ns = no significance, \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$  and \*\*\*\* $p \leq 0.0001$ ).  $n=3$  for SMN $\Delta$ 7 mice,  $n=4–5$  for Smn2B/- mice. Error bars represent mean  $\pm$  SEM.

**Additional file 4: Supplementary Figure 4.** Comparison of intrinsic muscle properties in four mouse models of SMA. The colour gradient legend describes seven classification categories of neuromuscular junction pathology according to the percentage of fully occupied motor endplates in a muscle at disease end-stage (see methods section for more details). The tables shows investigated muscles coloured according to its classification categories by percentage of fully occupied endplates in the Smn -/-; SMN2 (Murray et al., 2008; Murray et al., 2010; Thomson et al., 2012), Taiwanese (Lin et al., 2016), SMN $\Delta$ 7 (Murray et al., 2008; Ling et al., 2012; Comley et al., 2016) and Smn2B/- (Murray et al., 2015) mouse model. (A) The table displays the muscles categorised by delayed synapsing (DeSyn) or fast synapsing (FaSyn). Note that for the LALC muscle, different results were observed in two independent studies (\* = (Murray et al., 2008); # = (Thomson et al., 2012)). (B) The table displays the muscles categorised by muscle fibre type (fast-twitch, mixed fibre types and slow-twitch). Note that for the AS, AAL, LALC and lumbrical muscles, different results were observed in two independent studies (\* = (Murray et al., 2008); # = (Thomson et al., 2012); + = (Comley et al., 2016); ° = (Ling et al., 2012)).

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#### Authors' contributions

VW: conceptualisation and design of the experiments in Figs. 1, 2, 3, 4 and 5, acquisition, analysis and interpretation of the data for Figs. 1, 2, 3, 4 and 5, assembly of all figures and writing of the manuscript. IM: conceptualisation and design of the experiments in Figs. 5 and 6, acquisition and analysis of the data for Figs. 5 and 6, critical revision of the manuscript. EH: supervision of IM, conceptualisation of the experiments in Figs. 5 and 6, critical revision of the

manuscript, and acquisition of the funding. LM: conceptualisation, design and management of the project, supervision of VW, acquisition of the funding, critical revision of the manuscript. The authors read and approved the final manuscript.

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#### Availability of data and materials

The SMN $\Delta$ 7 mouse model RNA-seq dataset supporting the conclusions of this article is available in the Gene Expression Omnibus (GEO) accession number GSE115706. The Smn2B/- mouse model RNA-seq datasets supporting the conclusions of this article is available from the corresponding author (LMM) on reasonable request.

#### Declarations

##### Ethics approval and consent to participate

All applicable institutional and national guidelines for the care and use of animals were followed. All animal procedures were conducted in accordance with the guidelines set out by the University of Edinburgh and the UK Home office.

##### Consent for publication

Not applicable.

##### Competing interests

The authors declare that they have no competing interests.

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## 7.2. Appendix 2: Human Molecular Genetics paper – co-author



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Original Article

# Motor unit recovery following Smn restoration in mouse models of spinal muscular atrophy

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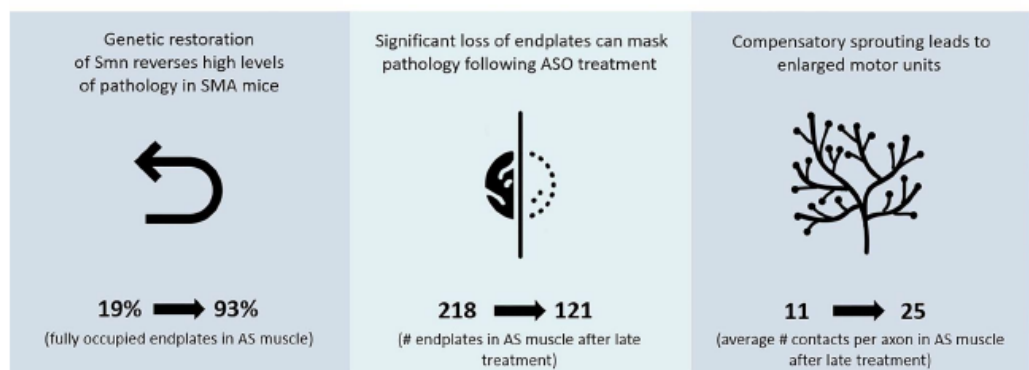
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### Abstract

Spinal muscular atrophy (SMA) is a childhood motor neuron disease caused by anomalies in the SMN1 gene. Although therapeutics have been approved for the treatment of SMA, there is a therapeutic time window, after which efficacy is reduced. Hallmarks of motor unit pathology in SMA include loss of motor-neurons and neuromuscular junction (NMJ/s). Following an increase in Smn levels, it is unclear how much damage can be repaired and the degree to which normal connections are re-established. Here, we perform a detailed analysis of motor unit pathology before and after restoration of Smn levels. Using a Smn-inducible mouse model of SMA, we show that genetic restoration of Smn results in a dramatic reduction in NMJ pathology, with restoration of innervation patterns, preservation of axon and endplate number and normalized expression of P53-associated transcripts. Notably, presynaptic swelling and elevated Pmaip levels remained. We analysed the effect of either early or delayed treatment of an antisense oligonucleotide (ASO) targeting SMN2 on a range of differentially vulnerable muscles. Following ASO administration, the majority of endplates appeared fully occupied. However, there was an underlying loss of axons and endplates, which was more prevalent following a delay in treatment. There was an increase in average motor unit size following both early and delayed treatment. Together this work demonstrates the remarkably regenerative capacity of the motor neuron following Smn restoration, but highlights that recovery is incomplete. This work suggests that there is an opportunity to enhance neuromuscular junction recovery following administration of Smn-enhancing therapeutics.

### Graphical Abstract

#### Motor Unit Recovery Following Smn Restoration in Mouse Models of Spinal Muscular Atrophy



Comley et al

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## Introduction

Spinal muscular atrophy (SMA) is a childhood motor neuron disease caused by mutations and deletions within the SMN1 gene (1). Due to a second partially functional copy of the gene, termed SMN2, which can exist in a variety of copy numbers, the severity of the disease varies. In its most severe form, SMA has an onset of under 6 months, with a life expectancy of ~2 years. A number of therapeutics have recently been approved for the treatment of SMA that increase SMN protein levels, including an antisense oligonucleotide (ASO), a small molecule drug and viral gene therapy (2–5). Thus far, treatments have demonstrated remarkable efficacy, significantly slowing decline or greatly enhancing motor ability and lifespan in treated patients. However, we do not yet know the long-term prognosis following these treatments. It is likely that long-term outcomes will be closely linked to the amount of damage at the point of treatment and the degree to which motor neuron pathology can be reversed.

Preclinical studies have shown that there is a dramatic reduction in therapeutic efficacy when treatment is delayed (6–9). This therapeutic time window has been investigated in mouse models, and a delay in treatment of 1 or 2 days can result in a dramatic decrease in life expectancy (10–12). It is important to understand why a temporal window exists and develops strategies that extend this.

Although other tissues have been implicated in SMA pathology, motor neurons remain the primary targets (13) with pathology at the neuromuscular junction (NMJ) preceding motor neuron loss (14). Importantly, not all muscles appear to be equally affected, with high levels of NMJ loss observed in some muscles, whereas other muscles remain intact until late stages of disease (15–18). Although the patterns of selective vulnerability are highly stereotyped, and there are important parallels between patients and mouse models, the reasons for this underlying selective vulnerability remain unclear.

Early treatment with a Smn-inducing compound appears to reduce NMJ defects, with efficacy varying by the route of administration and level of Smn achieved (10–12,19–24). Improvements were seen in various aspects of NMJ pathology including increased quantal content, decreased neurofilament (NF) accumulation and increased endplate maturation. However, it is unclear whether early treatment prevents the NMJ defects from manifesting or corrects pathology that has already commenced.

It is well established that the peripheral nervous system is capable of significant regeneration. This is observed following an acute peripheral nerve injury, or as ongoing remodelling and compensation occurring during progressive peripheral neuropathies (25). Indeed, injured axons are able to regenerate and reinnervate denervated muscle. Intramuscular axons are also able to grow new branches from internodes (nodal sprouts) or from the synaptic terminal (terminal sprouts) to

reinnervate endplates which have become denervated. These processes of compensation are major modifiers of disease time course. Indeed, extremely large motor units have been observed in patients, particularly with milder forms of SMA, where the few remaining motor neurons have sprouted and expanded their territory to compensate for the loss of their neighbours (26,27). However, it is unclear how motor unit expansion affects the overall health of the motor neuron, and it has been suggested that motor unit enlargement correlates with an increase in oxidative stress, which can affect the longevity of the cell (25).

It is currently unclear how pathology of the motor neuron is affected by restoration of Smn levels. For example, how much damage can be repaired effectively? Does restoration of Smn levels allow normal innervation patterns to be restored and is there a critical threshold of damage which occurs, which cannot be compensated for, that may explain the therapeutic time window in SMA?

Here, we have undertaken a detailed study of motor unit recovery following restoration of Smn levels. Using an Smn inducible mouse model of SMA, we show that at P4 [a time point where treatment with tamoxifen causes > 50% of mice to live over 200 days (11)], there are already high levels of NMJ pathology, including denervation, presynaptic swelling and an increase in transcripts associated with the P53 signalling pathway. Analysis of the pathology at P10 following Smn restoration at P4 reveals a remarkable recovery of the neuromuscular system within 6 days, suggesting that much of the pathology observed at P4 is rapidly reversible. Importantly, this recovery was achieved by reinnervation of denervated endplates and resumption of normal motor unit size, correlating with the predicted benefit to phenotype and lifespan following P4 rescue in this mouse model. To further investigate the basis for the effective therapeutic time window using a more clinically relevant approach, we treated 'delta7' mice (Smn<sup>-/-</sup>; SMN2<sup>+/+</sup>; SMNΔ7<sup>+/+</sup>) with a SMN-inducing ASO by intracerebral injection P1 (associated with a significant extension in lifespan) or P6 (associated with a limited extension in lifespan). Both early and late treatment with ASO led to very low levels of denervation. However, since there was also a reduction in total endplate number, the reduction in the number of denervated endplates can be partly attributed to the loss of these endplates rather than reinnervation. In moderately and severely affected muscles, there was also a loss of intramuscular axons and an increase in average motor unit size, which was more widespread following P6 treatment.

Collectively, this work highlights that rescue of the neuromuscular system is possible following restoration of SMN levels. However, it is incomplete and dependent on the method of treatment and the time point at which treatment is initiated. The long-term effects of incomplete rescue need to be further investigated to

better understand how to support patients undergoing treatment for SMA.

## Results

### There are significant levels of NMJ pathology at P4 before *Smn* restoration in *Smn<sup>Res/Res</sup>* mice

*Smn* inducible model mice (*Smn<sup>Res/Res</sup>; SMN2; SMN<sup>Δ7</sup>; Cre<sup>ERT</sup>*) have been described previously (11). They carry the *Smn<sup>Res</sup>* allele on the 'delta7' background. The *Smn<sup>Res</sup>* allele essentially functions as a null allele in the non-induced state, resulting in a severe SMA phenotype like the typical delta7 model. Administration of tamoxifen induces *Smn* expression, demonstrating that early induction significantly extends survival with over 50% of mice living over 200 days when *Smn* is induced at P4. It is unclear whether treatment at P4 prevents pathology manifesting, or whether regeneration/repair is required. To determine how much damage has been done to the neuromuscular system at this time point, we assessed phenotype and neuromuscular pathology at P4.

In agreement with previous work (11), in untreated mice at P4, we observed a significant reduction in body weight and motor deficits, as evidenced by an inability to self-right (Fig. 1A and B). We next quantified NMJ pathology in three muscles which have previously been shown to be vulnerable in other mouse models of SMA (17,28): auricularis superior (AS), transversus abdominis (TVA) and levator auris longus caudal band (LALC). This revealed high levels of denervation in all three muscles, with a significant increase in the percentage of partially occupied and vacant endplates, with only 19.7 (LALC)–55.9% (TVA) endplates remaining fully occupied (Fig. 1C and D). In those terminals which remained fully occupied, we saw a significant increase in presynaptic swelling, as evidenced by a significant increase in the percentage of NMJs with mild, moderate and severe swelling in all three muscles (Fig. 1E).

Previous work has shown that an increase in transcripts associated with the P53 signalling pathway correlates with the onset of NMJ pathology (14,29). We therefore quantified the levels of P53 and three transcripts known to be downstream of P53 (*Fas*, *Pmaip* and *Cdkn1a*) in spinal cord of P4 *Smn<sup>Res/Res</sup>* mice. This revealed a significant increase in all four transcripts (Fig. 1F).

Collectively, this work reveals that at P4, a time point when induction of *Smn* levels can confer a significant extension in life span, there are already significant motor deficits, high levels of NMJ pathology in at least three vulnerable muscles and activation of the P53 signalling pathway in the spinal cord.

### Restoration of *Smn* levels at P4 ameliorates NMJ pathology and reverses activation of P53 signalling pathway

Our analysis at P4 revealed significant neuromuscular pathology at P4. We next investigated the extent to which this pathology is reversible, and the time frame in

which this happens. Tamoxifen was administered by oral gavage at P4 (75 mg/kg) to induce full length expression of *Smn* in *Smn<sup>Res/Res</sup>; SMN2; SMN<sup>Δ7</sup>; Cre-ER<sup>+/+</sup>* mice (herein referred to as 'Rescue'). *Smn<sup>Res/Res</sup>; SMN2; SMN<sup>Δ7</sup>* mice were used as non-induced SMA mice (herein referred to as 'SMA') and *Smn<sup>+/+</sup>; SMN2; SMN<sup>Δ7</sup>; Cre-ER<sup>+/+</sup>* or *Smn<sup>+/+</sup>; SMN2; SMN<sup>Δ7</sup>; Cre-ER<sup>-/-</sup>* were used as non-SMA controls (herein referred to as 'Control'). Assessment of body weight revealed a trend towards an increase at P6 and P8, which was statistically significantly compared with 'SMA' mice, and indistinguishable from 'Control' mice by P10 (Fig. 2A). A significant improvement in motor performance was noted by P6, as evidenced by a decrease in the time taken to right from a prone position in 'Rescue' mice compared with SMA (Fig. 2B). Analysis of NMJ pathology revealed a dramatic reduction in NMJ loss compared with the same measurements taken at P4 and 'SMA' mice at P10 (Fig. 2C–F). Over 93, 87 and 95% of endplates were classified as fully occupied in AS, TVA and LALC, respectively, compared with 19, 57 and 29% in 'SMA' mice at P10. There was a significant but incomplete rescue in presynaptic swelling in the TVA and LALC muscles, but no significant improvement in the AS muscle. There was no significant change in endplate size in either AS, TVA or LALC in 'Rescue' mice compared with control or SMA (Fig. 2F).

Since q-RT-PCR analysis revealed a significant increase in P53 and downstream transcripts at P4, we investigated whether these changes were reversible. We observed a significant reduction in *Cdkn1a* and *Fas* in 'Rescue' mice compared with 'SMA', although *Pmaip* levels remained elevated (Fig. 2G). Collectively, this analysis demonstrated that many, but not all, aspects of neuromuscular pathology evident in a mouse model of SMA are reversible within a 6-day time frame.

### Motor unit size is preserved following restoration of *Smn* levels at P4 in *Smn<sup>Res/Res</sup>* mice

Data presented in Figure 2 suggest that innervation can be restored following induction of full length *Smn*. However, it remained unclear whether the majority of endplates appear fully occupied because denervated muscle fibres have been reinnervated, or because denervated endplates have been lost. To address this question, we quantified total endplate numbers in the LALC muscle. This muscle was chosen as it has a well-characterized anatomy and is small and thin enough to whole mount, allowing analysis of the entire muscle without the need for sectioning. Previous work has revealed a consistent endplate band in LALC, at the medial side of the muscle, denoted C2 (17,30). Quantification of total endplate number in C2 revealed no significant difference between 'Control' and 'Rescue' mice, suggesting that the increase in endplate occupancy was a result of reinnervation rather than loss of denervated endplates (Fig. 3). Interestingly, there was a significant decrease in the number of endplates in the LALC muscle in 'SMA' mice compared with both control and rescue, suggesting that

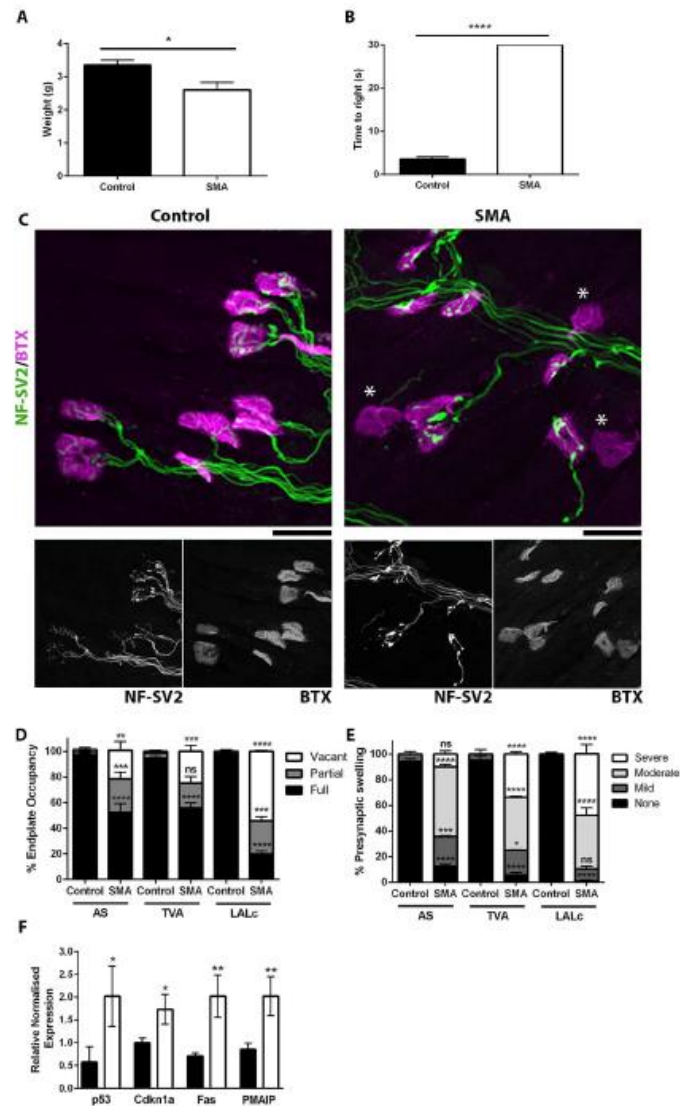


**Figure 1.** There is significant NMJ pathology at P4 in *Smn<sup>Res/Res</sup>* mice before *Smn* restoration. (A, B) Bar chart (mean $\pm$ SEM) showing significant decrease in body weight (A) and significant increase in time taken to self-right (B) in SMA (*Smn<sup>Res/Res</sup>;SMN2;SMN $\Delta$ 7*) mice compared with control (*Smn<sup>+/-Res</sup>;SMN2;SMN $\Delta$ 7* and *Smn<sup>+/-+</sup>;SMN2;SMN $\Delta$ 7*) at P4. (C) Images showing neuromuscular junctions (NMJs) in the TVA muscle labelled with antibodies against NF and SV2 (green) and BTX (magenta) in control and SMA mice at P4. Note presence of denervated endplates (BTX lacking presynaptic terminal) in SMA which does not occur in control. Scale Bar = 16  $\mu$ m. (D) Bar chart (mean $\pm$ SEM) showing percentage of fully occupied, partially occupied and vacant endplates in AS, TVA and Levator Auris Longus Caudal (LALc) muscles in control and SMA mice at P4. Note increase in vacant and partially occupied endplates in SMA and corresponding decrease in fully occupied endplates. (E) Bar chart (mean $\pm$ SEM) showing percentage of NMJs with no, mild, moderate or severe swelling in AS, TVA and LALc muscles in control and SMA mice at P4. Note increase in presynaptic swelling in SMA and corresponding decrease NMJs with no swelling. (F) Bar chart (mean $\pm$ SEM) showing increase in relative transcript levels of p53, Cdkn1a, Fas and Pmaip in the spinal cord of P4 SMA mice compared with control. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$  by Student's t test (A, B) or Mann-Whitney U test (D, E, F).  $N = 3/4$  mice, 6 muscles per genotype.

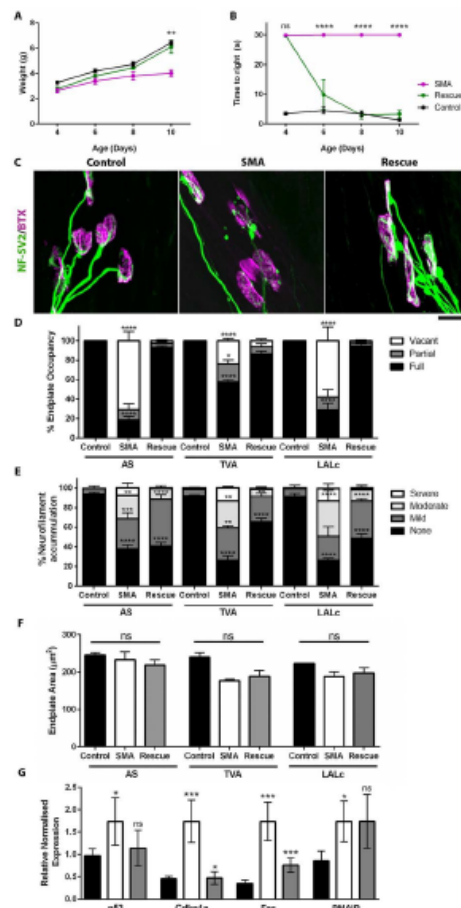


**Figure 2.** Restoration of *Smn* levels at P4 results in a significant rescue of NMJ pathology by P10. (A, B) Line chart (mean  $\pm$  SEM) showing body weight (A) and time taken to self right (B) in Rescue (*Smn<sup>Res/Res</sup>;SMN2;SMNΔ7;Cre<sup>+/+</sup>*), SMA (*Smn<sup>Res/Res</sup>;SMN2;SMNΔ7;Cre<sup>-/-</sup>*) and control mice at P4, 6, 8 and 10 days of age following treatment with Tamoxifen at P4. (C) Images showing neuromuscular junctions (NMJs) in the AS muscle labelled with antibodies against NF and SV2 (green) and BTX (magenta) in Rescue, Control and SMA mice at P10. Note presence of denervated endplates in SMA which was significantly reduced in Rescue mice. Scale bar = 20  $\mu$ m. (D) Bar chart (mean  $\pm$  SEM) showing percentage of fully occupied, partially occupied and vacant endplates in AS, TVA and Levator Auris Longus Caudal (LALC) muscles in Control, SMA and Rescue mice at P10. Stats show significant increase in percentage of fully occupied endplates in Rescue mice compared with SMA. (E) Bar chart (mean  $\pm$  SEM) showing percentage of NMJs with no, mild, moderate or severe swelling in AS, TVA and LALC muscles in Control, SMA and Rescue mice at P10. Stats show comparison of percentage of NMJs with no swelling, comparing Rescue to SMA. (F) Bar chart (mean  $\pm$  SEM) showing average endplate area in AS, TVA and LALC muscles in Control, SMA and Rescue mice at P10. (G) Bar chart (mean  $\pm$  SEM) relative transcript levels of P53, Cdkn1a, Fas and Pmaip in the spinal cord of P10 Control, SMA and Rescue mice. Stats show SMA versus control and Rescue versus SMA. Note significant reduction in Cdkn1a and Fas in Rescue Mice compared with SMA compared with control. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$  by two-way

endplates which remain denervated are lost (Fig. 3). This finding reveals that assessing NMJ pathology by occupancy count alone, a practice which is widespread in the field of neuromuscular disease, can dramatically underestimate the levels of degeneration present.

Data thus far demonstrate the requirement for significant regeneration following *Smn* restoration. Such regeneration can be achieved via terminal sprouting and motor unit enlargement from remaining motor neurons, or via regeneration of degenerating axons. Although the former would correlate with an increase in the size of the motor unit, the latter would result in a normal motor unit size. To distinguish between these possibilities, we have quantified axon number and extrapolated average motor unit size. Since axon number can vary along the length of a nerve, this analysis was performed in the AS muscle, a muscle in which the point at which two incoming bundles of axons cross over in a highly consistent manner. This allows us to quantify axon number at a consistent location in all muscles.

Quantification of axon number revealed that although there was a significant loss of axons in the AS muscle in 'SMA' mice, the number of axons in the 'Rescue' mice was not different to control levels (Fig. 4A and B). By dividing the number of innervated NMJs by the number of axons, we extrapolated the average motor unit size. This revealed that although there was a significant increase in the average motor unit size in 'SMA' mice, in Rescue mice, average motor unit size was not significantly different to 'Control' levels (Fig. 4D).

Collectively, these data show that restoration of *Smn* levels can result in remarkable protection of the motor unit, even after significant loss of NMJs and pathology within the motor unit. In addition, if *Smn* levels are restored before significant motor neuron loss, re-innervation can re-establish a normal motor unit size, correlating with a dramatic extension in lifespan.

### Delayed treatment with an ASO targeting SMN2 leads to widespread loss of axons and endplates

To investigate motor unit recovery using a more clinically relevant approach, we compared motor unit pathology following restoration of *Smn* levels using ASO targeting Element 1 of SMN2 [ASO<sup>E1v1.11</sup>; (31)] in the delta7 SMA mouse model. Previous work has routinely shown a dramatic extension in lifespan following SMN induction, using both genetic and viral approaches, before P2, with median lifespan of over 200 days (10–12). In contrast, mice in which SMN levels are increased at P6 or later have a median lifespan of between 30 and 35 days (10–12). This indicates that important changes occur between P2 and P6 which limit the effective therapeutic time window. To allow equivalent time for regeneration, this analysis was performed 11 days after ASO treatment i.e. P12 in P1-treated mice and P17 in P6-treated mice. As in other mouse models

of SMA, intermuscular variability to pathology is a prominent feature in this mouse model, with different muscles following distinct time courses of degeneration. Therefore, to get a thorough overview of motor neuron recovery, we analysed three differentially vulnerable muscles: levator auris longus rostral (LALr, resistant), adductor auris longus (AAL, moderately affected) and AS (severely affected) (Supplementary Material, Fig. S1). All muscles benefit from being small, thin and flat allowing a comprehensive analysis of innervation patterns.

To determine whether levels of innervation correlated with day of ASO administration, we first quantified the percentage of fully occupied endplates 11 days after P1 or P6 ASO administration, respectively (Fig. 5B–D). As expected, all endplates in the LALr were fully occupied. Interestingly, most endplates in AAL and AS muscles were also fully innervated after both P1 and P6 treatment, with the exception of a significant number of partially occupied and denervated endplates remaining in the AS following P1 treatment (Fig. 5D). Intriguingly, analysis of degree of NF accumulation revealed a similar trend (Supplementary Material, Fig. S2).

Since quantification of the percentage of fully occupied endplates alone can mask axonal and endplate loss (c.f. Fig. 4D), we next quantified axon and endplate numbers following P1 and P6 ASO treatment. Once again, there was no significant difference in the number of axons or endplates in the LALr muscle, verifying that pathology has not been masked by endplate fading or sprouting, and this muscle is truly resistant (Fig. 5E and H). In the moderately affected AAL muscle, although P1 treatment preserved axon and endplate number, following P6 treatment, there was a significant decrease in axon number (Fig. 5F and I). In the severely affected AS muscle, there was a significant loss of both axons and endplates following both P1 and P6 treatment. This work highlights the importance of considering multiple muscles and demonstrates each muscle follows its own degenerative timeline. It suggests that although a P1 intervention is adequate to rescue moderately affected muscles, for severely affected muscles, significant damage is present at P1 which was not reversed by ASO treatment.

#### Delayed treatment with a Smn-inducing ASO results in a significant increase in motor unit size in a range of muscles

Since we observed a decrease in axon number in the absence of denervated endplates in the moderately affected AAL, we hypothesized that motor unit sprouting was compensating for some denervation following ASO administration. Qualitative analysis of AS muscles 12 days after ASO treatment revealed widespread evidence of sprouting (Fig. 6A). Indeed, in some muscles

analysed only one or two motor units remained, allowing the entire motor unit to be traced. Examples of large motor units were noted, with size frequently around double the average motor unit size of controls (Fig. 6Ai). There was widespread evidence of terminal sprouting, where new axonal growth extends from the presynaptic terminal to reinnervated endplates which presumably had become denervated (Fig. 6Aii–iii).

To investigate the effect of this compensatory sprouting on the motor unit, we extrapolated average motor unit size by dividing the total number of innervated endplates by the number of axons entering the muscle. As expected, motor unit size was stable in the LALr muscle, (Fig. 6B). In the AAL muscle, although P1 ASO treatment preserved motor unit size, delayed P6 treatment led to a 2.2-fold increase in average motor unit size (Fig. 6C). In the vulnerable AS muscle, motor unit size was significantly increased following both P1 and P6 treatment (Fig. 6D). Collectively, these data demonstrate that following delayed administration of the ASO, the neuromuscular system is capable of dramatic repair, with considerable compensatory sprouting resulting in the reinnervation of denervated endplates in a relatively short time frame. However, regeneration results in a dramatic increase in motor unit size, which correlates with a poor phenotypic outcome.

#### Discussion

The data presented here highlight the enormous regenerative capacity of the motor neuron following Smn restoration. Its impressive ability to recover from high levels of denervation in a comparatively short time frame is very encouraging, especially for patients treated after symptom onset. Preserving axon and endplate number and retaining a normal motor unit size all correlated with a dramatic extension in lifespan. Importantly, due to the previously unappreciated rapid loss of denervated endplates, the widely used technique of quantifying the percentage of fully occupied endplates significantly underestimates the levels of presynaptic degeneration. Our results also revealed that following ASO treatment and in untreated SMA mice, there was loss of axons and endplates and an increase in motor unit size, which was more prevalent following a delay in ASO administration. Investigating factors which can support large motor units throughout the lifespan of the individual may therefore represent an important therapeutic strategy for patients treated with a Smn-dependant therapeutic.

#### Extent of recovery at the NMJ

Results here highlight the remarkable regenerative capacity of the NMJ, but also reveal that some NMJ defects persist following Smn restoration. Understanding

ANOVA (A,B), one-way ANOVA with Tukey's post hoc test (F) or Kruskal–Wallis with Dunn's Post Hoc (D, E, G). N = 12/3/3 mice per Control/Rescue/SMA (A, B); N = 6/3/6 muscles per Control/SMA/Rescue (D–F) and N = 3 mice per genotype (G).

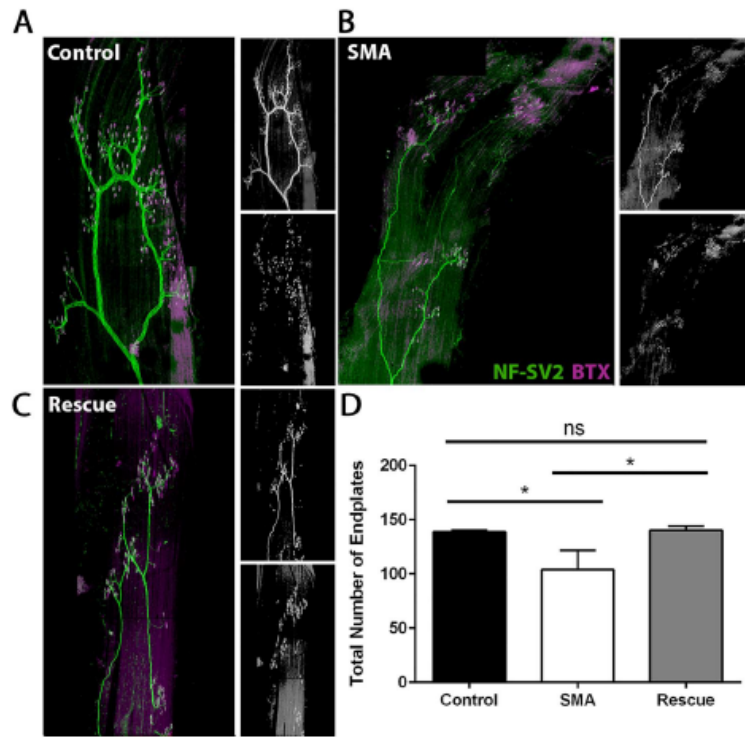


**Figure 3.** Recovery from denervation is due to reinnervation of denervated endplates. (A–C) Montages fluorescent micrographs showing C2 region of LALC muscle labelled with antibodies against NF and SV2 (green) and BTX (magenta) in Control (A), SMA (B) and Rescue (C) mice at P10. Scale Bar = 200  $\mu$ m. (D) Bar chart (mean  $\pm$  SEM) showing significant decrease in total endplate number in SMA compared with both Control and SMA, but no difference in total endplate number in Rescue versus Control. \* $P < 0.05$ ; ns, non-significant by Student's *t* test.  $N = 6/5/6$  muscles per Control/SMA/Rescue.

the impact of these anomalies upon NMJ function and motor neuron health will be critical. Following genetic restoration of *Smn* levels, presynaptic swelling at the neuromuscular junction remains, the impact of which is unknown. Presynaptic swelling has been reported in muscles which are apparently resistant to pathology, suggesting it has minimal impact upon physiology. However, since mouse models of SMA have a significantly curtailed lifespan, the impact of these defects over a prolonged period has not been evaluated. It will therefore be important to determine whether similar defects exist in a human population, whether they resolve in the long term and what impact they have upon motor neuron physiology over a prolonged period of time.

Following ASO administration, there was a loss of axons and endplates, which occurred in some muscles even when ASO was given at P1. The reason why rescue was less complete following ASO treatment, compared with the genetic rescue is unknown. It is possible that this is because of the higher *Smn* levels achieved in the genetic rescue; however, unfortunately, this was not something that we were able to assess. Regardless, this work highlights that following *Smn* restoration,

especially after ASO treatment, recovery is incomplete. Understanding the basis for these defects, and the reasons why they are not corrected, could be the basis for combinatorial therapeutics to aid NMJ recovery and aid the long-term motor neuron health following *Smn*-dependant therapeutic administration in SMA.

#### Denervation induced loss of motor endplates

Here we show that, following presynaptic pathology and a loss of presynaptic inputs, motor endplates are lost. Endplates initially appear visibly fainter following labelling with alpha-bungarotoxin (BTX), suggesting they are progressively disassembled following loss of presynaptic input. Previous work has often reported that although there is a significant increase in denervated endplates in the early symptomatic phases in mouse models of SMA, the severity of denervation does not increase as pathology progresses (16,17). The data presented here suggests that this could be due to the loss of endplates as they become denervated, rather than the cessation of denervation. The quantification of the percentage of fully occupied endplates is standard practise for assessing the degree of presynaptic

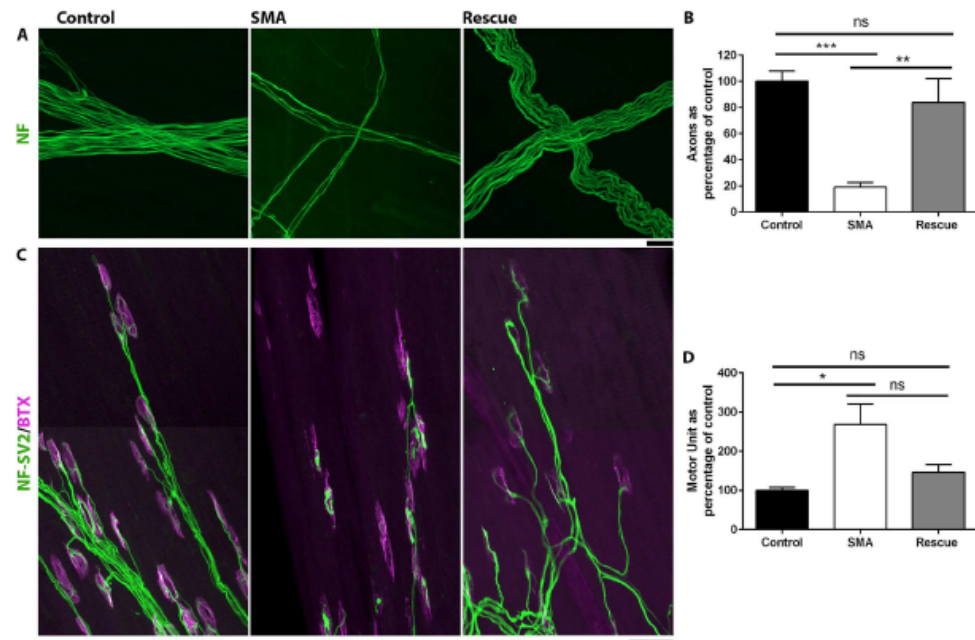


**Figure 4.** Smn restoration at P4 preserves axon number and motor unit size. (A, C) Images showing axon bundles (A) and neuromuscular junctions (NMJs) (C) labelled with antibodies against NF and SV2 (green) and BTX (magenta) in control, SMA and Rescue mice at P10. Scale bar = 20  $\mu$ m (A) and 50  $\mu$ m (C). (B, D) Bar chart (mean  $\pm$  SEM) showing number of axons (B) or average motor unit size (D) in AS muscle in Control, Rescue and SMA mice at P10. Note decrease in axon number and increase in motor unit size in SMA which was not seen in Rescue mice \* $P < 0.05$ ; \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , ns non-significant by Student's *t* test (B) or Mann-Whitney U test (C). *N* = 5/6/5 muscles per Control/SMA/Rescue.

degeneration occurring in SMA and other peripheral neuropathies. However, these current findings highlight how this can grossly underestimate, and even mask, levels of presynaptic degeneration. These data therefore invite careful consideration as to the methods used to quantify presynaptic loss so that it is accurately represented.

To our knowledge, the fading and loss of endplates in models of motor neuron disease have not been previously characterized. Endplate stability has been studied in experimentally denervated muscles, with significant remodelling of the AChRs, fading and loss of chronically denervated receptor clusters reported following nerve injury (32). However, Kang *et al.* also showed that persistent clusters of AChRs remain over 20 days after denervation. The loss and fading of AChR clusters in this study appear to occur at a much more rapid rate. This could be due to the comparatively immature status of endplates in mouse models of SMA (17,33), as the rate of AChR turnover is known to be dramatically increased during post-natal development (34,35). Alternatively, the rapid loss of endplates could indicate an intrinsic defect in AChR maintenance caused by a deficiency in Smn. Tight control of actin dynamics is thought to have an important role in stability and turnover of AChRs, particularly during postnatal development (35–38). Since defects

in the actin cytoskeleton have been widely reported in mouse models of SMA [e.g. (39–43)], it is tempting to speculate that this could contribute to the rapid loss of AChR clusters following denervation. Distinguishing these possibilities will be vital in determining the importance and translational relevance of AChR receptor loss following denervation.

#### Implications of motor unit enlargement in SMA

Due to sprouting from remaining axons, the average motor unit size can increase following Smn restoration. This was particularly evident following a delay in treatment. Motor unit enlargement has been previously described in SMA patients and in asymptomatic mouse models with reduced Smn levels. Although in the short term, an increase in motor unit size can compensate for denervation and slow disease progression, larger motor units are thought to encounter heightened levels of oxidative stress, because of their larger metabolic demand. Indeed, many individuals who experienced motor neuron loss due to acute polio infection in childhood have gone on to experience muscle weakness, fatigue and atrophy in adulthood, even in muscles which were apparently not affected during the acute infection (44,45). This has been termed post-polio syndrome and is thought to be caused by the premature aging and loss

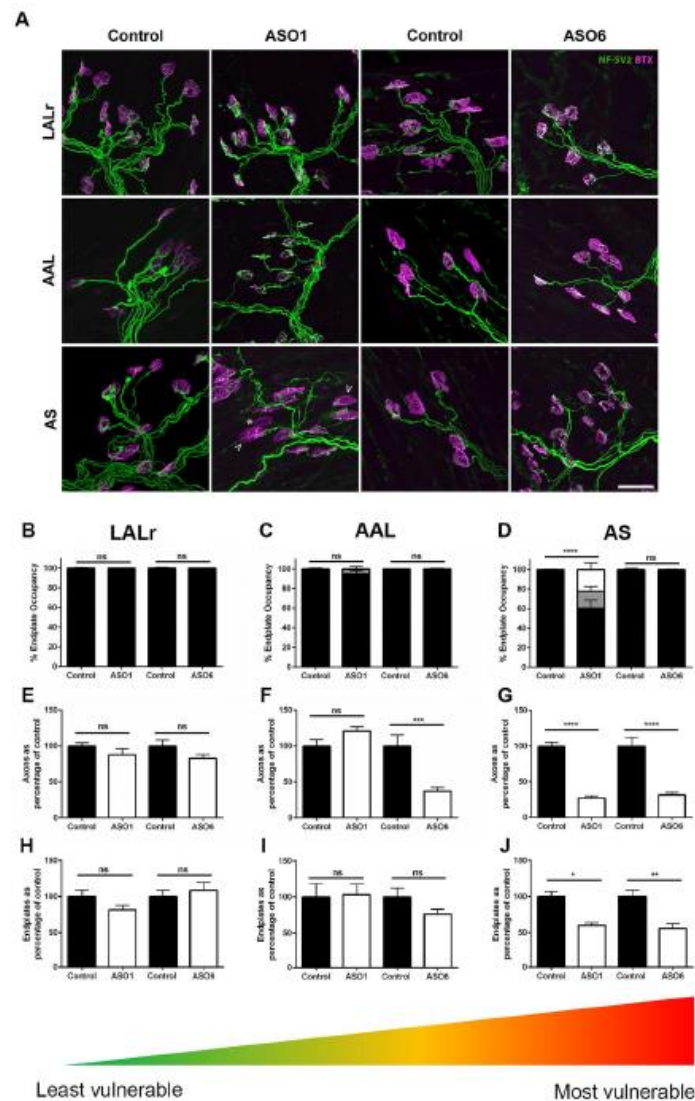


**Figure 5.** Delayed treatment with a Smn-inducing ASO leads to more widespread loss of axons and endplates. (A) Confocal images showing NMJs labelled with antibodies against NF and SV2 (green) and BTX (magenta) in *Smn* $\Delta$ 7 mice treated with ASO at P1 (ASO1) or P6 (ASO6) with their respective controls. Muscles used are the LALr (least vulnerable), AAL (moderately vulnerable) and AS (most vulnerable). Vacant and partially occupied endplates in the AS after early treatment (ASO1) are marked by an asterisk and arrow heads, respectively. Scale bar = 20  $\mu$ m. (B–D) Bar charts (mean  $\pm$  SEM) showing the percentage of fully occupied (black), partially occupied (grey) or vacant (white) endplates in P12 *Smn* $\Delta$ 7;*SMN2*;*SMN* $\Delta$ 7 mice treated with ASO at P1 (ASO1) or P6 (ASO6) compared with similarly treated controls. Muscles used are the LALr (least vulnerable), AAL (moderately vulnerable) and AS (most vulnerable). (E–J) Bar charts (mean  $\pm$  SEM) showing the number of axons (E–G) and innervated NMJs (H–J) in the three muscle groups in P12/P17 *Smn* $\Delta$ 7;*SMN2*;*SMN* $\Delta$ 7 mice following early (ASO1) or delayed (ASO6) treatment compared with controls. Note the significant loss of axons in the moderately vulnerable AAL muscle following delayed P6 treatment which was not seen in the early P1-treated cohort. Significant loss of both axons and endplates was seen in the severely vulnerable AS muscle following both P1 and P6 treatment. \*P < 0.05; \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001, ns non-significant by Mann–Whitney U test. N = 3–10 muscles per group.

of large motor units which underwent compensatory enlargement during childhood (45). This may have particular relevance to SMA patients treated with a

Smn-dependent therapeutic, and it will be important to monitor motor ability throughout their lifespan and to consider the likely outcome for enlarged motor units

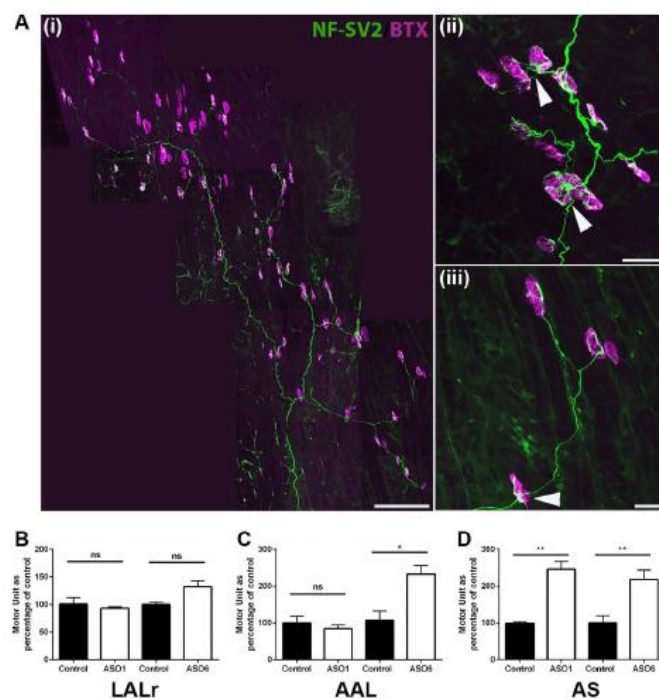


**Figure 6.** Axonal sprouting leads to increased motor unit size in both vulnerable and moderately resistant muscles following ASO treatment. (A) Confocal images showing NMJs labelled with antibodies against NF and SV2 (green) and BTX (magenta) in the AS muscle of a P12 *Smn*<sup>-/-</sup>;SMN2;SMNΔ7 mouse treated with ASO at P1. Note presence of large motor units (Ai) and evidence of significant presynaptic remodelling and terminal sprouting (Aii and Aiii) respectively. Scale bar = 150 μm (Ai), 20 μm (Aii and Aiii). (B, C, D) Bar charts (mean ± SEM) showing average motor unit size in LALr, AAL and AS muscles of mice treated with ASO at P1 (ASO1) or P6 (ASO6) compared with similarly treated controls. Note that the significant loss of axons in the moderately vulnerable AAL muscle is masked by compensatory sprouting leading to enlarged motor units (B). \**P* < 0.05; \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001, ns non-significant by Mann-Whitney U test. *N* = 3–10 muscles per group.

and how to best support them throughout the lifespan of the individual.

## Material and Methods

### Mouse breeding and maintenance

*Smn*<sup>+/<sup>Res</sup></sup>;SMN2;SMNΔ7;Cre<sup>ERT</sup> mice (strain 008783, mixed C57BL/6;FVB/N;129 genetic background) were purchased from Charles River and maintained as *Smn*<sup>+/<sup>Res</sup></sup>;SMN2;SMNΔ7;Cre-ERT<sup>+/-</sup> × *Smn*<sup>+/<sup>Res</sup></sup>;SMN2;SMNΔ7 breeding pairs at the animal facilities at the University of Edinburgh. *Smn*<sup>+/-</sup>;SMN2<sup>+/-</sup>;SMNΔ7<sup>+/-</sup> (Strain 005025, Strain FVB/N) were maintained as breeding pairs at the University of Missouri. All genotyping was performed using standard PCR protocols on DNA extracted from an ear punch (for breeding pairs), tail tip (taken post-mortem for experimental mice) or tail sample (taken before tamoxifen treatment for tamoxifen treated mice). Mice over P10 were sacrificed by overdose of inhalation

anaesthetic or rising CO<sub>2</sub> and death was confirmed by exsanguination from the carotid artery. Mice under P10 were decapitated under terminal anaesthesia and death was confirmed by exsanguination from the carotid artery. All experiments were performed in accordance with the regulations set out by the UK Home Office (for animal experiments performed in the UK) or approved by the IACUC at the University of Missouri.

### Administration of tamoxifen

Tamoxifen (Sigma-Aldrich) was dissolved in corn oil to a concentration of 20 mg/ml. Tamoxifen solution was administered to litters of mice at P4 using oral gavage at a dose of 75 mg/kg. For Figures 2–4, all mice in a litter received tamoxifen. Cre-negative mice (i.e. *Smn*<sup>Res/Res</sup>;SMN2;SMNΔ7) were used as SMA model mice, as no recombination will take place, and mice carrying one copy of the Cre transgene (i.e. *Smn*<sup>Res/Res</sup>;SMN2;SMNΔ7;Cre-ERT<sup>+/-</sup>) were used as 'Rescue' mice.

### Antisense oligonucleotide treatment

P1 SMNΔ7 mice were injected with 4 μL of a 20 nM solution of E1<sup>V1.11</sup>, a Phosphordiamidate morpholino oligomer (PMO) ASO (31). The following primer sets were used: for the mouse *Smn* gene, mSmn-Wildtype (WT) forward (5'-tctgtgttcgtgcgtggtgacttt-3') and mSmn-WT reverse (5'-cccaccacctaagaaagcctcaat-3') and for the *Smn* knockout, SMN1-KO forward (5'-ccaacttaatcgcttgacgaca-3') and SMN1-KO reverse (5'-aagcgagtggaacatggaaatcg-3'). Intracerebroventricular (ICV) injections were performed on P1 and the injection site was ~0.25 mm lateral to the sagittal suture and 0.50–0.75 mm rostral to the neonatal coronary suture. The needles were inserted perpendicular to the skull surface using a fiber-optic light (Boyce Scientific Inc.) to aid in illuminating pertinent anatomical structures. Needles were removed after 10 s of discontinuation of plunger movement to prevent backflow. Treated animals were placed in a warmed container for recovery (5–10 min) until movement was restored.

### Motor testing

The 'time to right' test was carried out on mice younger than P10. Mice were placed on their back and the time it took for them to right themselves (as defined by the placement of all 4 paws on the testing surface) was recorded, with a maximum of 30 s and an average of three attempts.

### NMJ labelling

Muscles were immediately dissected from recently sacrificed mice and fixed in 4% paraformaldehyde (Electron Microscopy Science) in PBS for 15 min. Post-synaptic AChRs were labelled with α-BTX (1:250; tetramethylrhodimine conjugate, Life Technologies) for 2 h. Muscles were permeabilized in 2% Triton X-100 in phosphate buffered saline (PBS) for 30 min, then blocked in 4% bovine serum albumin/1% Triton X-100 in PBS for 30 min before incubation overnight in primary antibodies [NF, 1:50 (2H3)—Developmental Studies Hybridoma Bank; synaptic vesicle protein 2, 1:100 (SV2)—Developmental Studies Hybridoma Bank] and visualized with secondary antibodies (AlexaFluor-488 rabbit anti-mouse at 1:250, Jackson). Muscles were then whole-mounted in Mowiol (Sigma).

The percentage of fully occupied endplates was determined by classifying each endplate in a given field of view either fully occupied [presynaptic terminal (SV2 and NF) completely overlies endplate (BTX)], partially occupied [presynaptic terminal only partially covers endplate (BTX)] or vacant (no presynaptic label overlies endplate). For presynaptic swelling, all NMJs were classified as having no swelling, mild (evidence of some axonal swelling and/or presynaptic swelling that does not obscure the end plate), moderate (evidence of clear axonal swelling and significant presynaptic swelling that is beginning to obscure the end plate) or severe (significant and obvious swelling along the length of the axon and severe swelling

around the presynaptic terminal, which obscures the endplate). Endplate size was quantified using ImageJ software. At least three fields of view were analysed per muscle totalling > 50 endplates per muscle. Endplate number was assessed by counting the number of AChR clusters within an anatomically consistent area of the muscle (LALC, C2; AS and AAL, entire medial endplate band). NMJ numbers were assessed by counting the number of fully or partially innervated endplates in an anatomically distinct area of the muscle. Only muscles which were present in their entirety with no signs of damage or disruption to innervation patterns were used for these measurements. Axon number was counted in a consistent region of each muscle (the point at which axons cross in the AS and 400 μm from the main endplate band in the AAL). Average motor unit size was calculated by dividing the total number of innervated NMJs in a given muscle by the number of axons in the same muscle. All quantification was performed with researcher blind to genotype of material.

### q-RT-PCR

RNA was extracted using a micro RNeasy kit (Qiagen) and 1 μg of RNA was used to perform reverse transcriptase using the RT<sup>2</sup> First Strand kit (Qiagen). SYBR green-based Q-RT-PCR was performed using preoptimized primers purchased from Qiagen. Amplification was performed using KAPA SYBR fast universal PCR mastermix as per manufacturer instructions on a BioRad CFX connect real-time PCR detection system. Relative gene expression was calculated using the 2<sup>-ΔΔCt</sup> formula (46). Relative levels are expressed normalized to GusB (F: GGCTGGTGACCTACTGGATT; R: TTGGCACTGGGAACCTGAAGT) and YWHAZ (F: TTGATCCCCAATGCTTCGC; R: CAGCAACTCGGCCAAGTAA).

### Supplementary material

Supplementary Material is available at HMG online.

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