Potentiation of neuromuscular transmission as a therapeutic strategy to improve motor function in spinal muscular atrophy

by

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Spinal Muscular Atrophy (SMA) is a genetic disease caused by a null mutation of the SMN1 gene. Loss of SMN1 results in low levels of a protein called Survival of Motor Neuron (SMN), which is a protein that is critical for neuromuscular development. The first FDA-approved treatment for SMA utilizes intrathecal injections of an antisense oligonucleotide (ASO) to increase expression of SMN. Despite the immense excitement for this treatment, however, preliminary clinical observations and studies in SMA mouse models indicate persistent neuromuscular weakness, which reveals the need for an additional symptomatic treatment that targets neuromuscular function. Thus, supplemental strategies are required to address the neuromuscular deficits that remain after ASO therapy. In our preclinical investigations, we have tested a calcium channel gating modifier (GV-58), which significantly increases transmitter release from weakened motor nerve terminals, in combination with a potassium channel blocker (3,4-diaminopyridine; 3,4-DAP).

3,4-DAP is a drug commonly prescribed to patients with specific motoneuron diseases, but scientists have debated channel selectivity and concentration-response effects. To address these questions, my colleagues and I have characterized the mechanism of action of 3,4-DAP at neuromuscular junctions across two species (Chapter 2). Our results have provided novel insight into the concentration-dependent effects of 3,4-DAP on presynaptic voltage-gated potassium

channels, as well as physiological effects on presynaptic action potentials and the magnitude of transmitter released.

We next provide proof of principle that 3,4-DAP can be combined with GV-58 to increase strength and improve neuromuscular function in an SMA model mouse (Chapter 3). We have found that GV-58 alone is an excellent therapeutic candidate to restore neuromuscular function and increase strength in more mild forms of SMA, but severe forms of SMA optimally benefit from GV-58 combined with 3,4-DAP. The preclinical investigations contained within this dissertation provide the initial research necessary to explore the efficacy of a novel treatment that complements current approaches by addressing persistent deficits after ASO therapy.

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Preface

I want to thank everyone who has helped me on this journey – my committee members, my undergraduate and post-undergraduate mentors, my graduate program and the entire community it comprises. I thank the many friends across many states and many countries that have encouraged me to keep going and to do better, or encouraged me to stop, take a breath, and sip a beer when I needed a moment of restoration. I thank the entire Meriney lab – my science family – for the endless help, the constant friendship, our shared "inner peace", and the limitless support in science and non-science affairs. Yinz have been wonderful and I hope we move the lab to the beach soon. I also thank my family for their love of me, love of beauty and fashion, love of gambling and card games ("statistics" lessons), and love of all the lopsided and decrepit creatures that have been a part of my menagerie. Finally, I want to say thanks to Gregory, who refuses to live without me despite 4 years and 1,344 miles of distance.

1.0 Introduction

Spinal muscular atrophy (SMA) is a heritable and predominantly neuromuscular disorder that is characterized by the slowly progressive and irreversible death of lower α-motoneurons in the anterior horn of the spinal cord. First documented in 1891 and 1893 respectively by Guido Werdnig and Johan Hoffmann, (Hoffmann, 1893; Werdnig, 1891), physicians and researchers over the subsequent century documented the clinically heterogeneous, seminal pathology of motoneuron degeneration, the symmetrical and predominantly proximal and axial muscle weakness, and familial association of the disease presently known as SMA. Because the clinical heterogeneity comprises a spectrum of phenotypic severity (ranging from severe somatic and respiratory paralysis to minor gait abnormalities), SMA is a complex manifestation of a relatively straightforward problem. SMA is monogenic in origin, caused by the homozygous functional loss or deletion of a gene critical for the viability of motoneurons (aptly named Survival of Motor Neuron, or *SMN1*). The range in symptoms can generally be attributed to the expression of a second genetic homolog, *SMN2*, which partially compensates for the loss of *SMN1*.

Gradual death of motoneurons results in a widespread loss of motor units and the downstream atrophy of skeletal muscle fibers. Consequently, the progressive decline in motor function, most often during the first few years of life, is a hallmark for symptomatic diagnosis. Cognition, intellect and affective states are preserved in individuals affected by SMA (though with the caveat that assessment is difficult in infants with extremely severe forms of SMA, as they experience significantly shortened lifespans). Additionally, some loss of sensory neuron function has been observed in patients with the most severe form of SMA. The complex medical needs of patients diagnosed with SMA diminishes quality of life (Tilton, Miller, & Khoshoo, 1998) and

increases stress experienced by patients and their caregivers (Gontard, Rudnik-Schöneborn, & Zerres, 2012; Lamb & Peden, 2008; Qian et al., 2015).

Despite a prevalence of ~1:10,000 births (Prior, 2008), SMA has historically been a relatively unknown disease. This obscurity was, in part, due to the lack of effective treatment; with the advent and availability of recent genetic approaches and screening of newborns for *SMN1* deletion/mutation, SMA is being clinically recognized more often and prior to symptom onset. The Federal Drug Administration approved the first therapy for SMA in December 2016, which has remarkably altered the natural history of the disease and challenges the traditional classification of patient outcomes. Since this first therapy debuted, several other genetically targeted approaches are in various stages of clinical development or have achieved approval for use in patients.

1.1 Clinical manifestation of spinal muscular atrophy: Description and classification of SMA types

The spectrum of SMA comprise heterogeneous phenotypes of motor impairment, spanning from extensive paralysis to mild constraints in motor function. In 1992, the International SMA consortium published a scale that is commonly used to classify the prognosis of a patient that was based on the age of symptom onset and highest level of motor function achieved or severity of motor decline (Munsat & Davies, 1992). The scale initially comprised SMA types 1-3, though subsequent modifications have added groups 0 and 4. Type 0 is associated with a perinatal onset and comprises the most significant motor impairment and rapid disease progression; in contrast, type 4 comprises relatively mild, slowly progressive muscle weakness appearing late into adulthood. These type categories were established to infer the prospective function and disease

progression of SMA affected individuals. Generally, types 0-1 are considered ‘non-sitters’, types 2 as ‘sitters’, and types 3-4 are ‘walkers’. However, with the advent and utilization of genetic therapies to modify disease severity, SMA patients may transcend single categorical classification over time. The prospective role of genetic therapies will be discussed in detail in later sections, and the following phenotype descriptions are based on the “natural” history of the disease (or as Dr. Victor Dubowitz, a pioneer of the SMA field, would argue, an "unnatural" history (Dubowitz, 2015)).

Some clinical manifestations of SMA are pathognomonic of several SMA types, including tongue fasciculation (indicative of lower motor neuron disease), progressive proximal to distal motor impairment affecting legs more than arms, and hyporeflexia of deep tendons. However, categorical types generally comprise distinct pathological features.

1.1.1 SMA type 0

The most severe form of SMA, type 0 (or sometimes documented as Type 1a) (Darras, 2015) is characterized by decreased movement *in utero* during the third trimester of gestation. If the child survives gestation, upon birth they may exhibit severe hypotonia, facial diplegia, weak or absent gag and suck reflexes, a weak cry, arthrygryphosis (limited joint contracture), digital necrosis, and extensive motor and sensory nerve deficits. Additionally, type 0 children experience a high incidence of congenital heart defects and, most notably, profound respiratory distress. Many patients exhibit a collapsed or bell shaped deformity of the chest coinciding with paradoxical breathing, suggestive of a lack of intercostal muscle opposition to diaphragmatic function (Beevor, 1902). Consequences of compromised respiratory function include an impaired cough that prohibits clearance of the lower airway, poor ventilation during sleep, developmental abnormalities

of the chest wall and lungs, and an increased susceptibility to respiratory infections. These sequelae exacerbate muscle weakness and compromise the integrity of lung parenchyma (Schroth, 2009). SMA type 0 children have a poor prognosis and typically die prior to 6 months of age.

1.1.2 SMA type 1

Patients that exhibit SMA symptoms at birth or by 6 months of age are considered type 1, also known as infantile-onset or Werdnig-Hoffman disease. SMA type 1 is the most common form of SMA, comprising 45-60% of SMA cases. Patients classified as type 1 are characterized by severe generalized muscle weakness and hypotonia. Muscle groups in the trunk, neck and proximal limbs experience weakness prior to distal limbs, and legs are more affected than arms. Gradually, muscle weakness evolves into paralysis, and infants present as floppy with symmetrical hypotonia and a lack of head control. SMA type 1 children are non-ambulatory, as they never achieve the ability to sit upright unaided, roll over, and never progress to advanced skills such as crawling or walking. As such, type 1 patients are dependent for mobility and self-care. Similar to type 0, patients with type 1 often exhibit a bell-shaped thorax, diaphragmatic breathing patterns, and respiratory distress. In addition to problems with breathing, type 1 patients also experience dysphagia (sucking and swallowing difficulties caused by muscle weakness), and for these reasons most severe SMA patients die of asphyxiation due to profound difficulties in breathing (Dubowitz, 1999) or due to aspiration pneumonia (Birnkrant et al., 1998).

Without mechanical intervention, 50% of children with SMA type 1 die by 1 year of age, and survival beyond 2 years is exceptional without invasive ventilation (Dubowitz, 1999). Benefits of ventilator support for type 1 children include a potential for prolonged lifespan, facilitation of at-home childcare, and improvements in chest wall deformities and lung development. However,

invasive ventilation for this classification of patients is controversial, and differences in standard health care practices across countries guide the use of this invasive care strategy. For example, according to a 2011 survey, physicians in the United States were more likely (25%) to recommend invasive ventilation than physicians from Commonwealth countries (U.K., Australia, Canada, etc.; 7%) (Benson et al., 2011). Hesitance to utilize invasive methods is due, in part, to the overall fragility and poor prognosis for type 1 patients. Use of gene therapy is likely to impact these factors, so further assessment is needed to understand the requirement of care practices after genetic intervention(s).

1.1.3 SMA type 2

Children with SMA type 2, or intermediate SMA, experience the onset of progressive weakness between 6 - 18 months of age, and represent approximately 20% of SMA cases. Unlike SMA type 1, type 2 patients achieve the ability to sit unaided, but fail to achieve independent standing. In addition, moderate to profound scoliosis (a consequence of weakened trunk muscles) is nearly universal in type 2 patients. Scoliosis can cause decreased lung capacity and affect respiratory and pulmonary function (Tsiligiannis & Grivas, 2012), and patients may require corrective surgery to improve quality of life (Garg, 2016). Similar to SMA type 1, motor weakness follows the same proximal to distal pattern, with a greater loss of function in legs than arms. However, after the initial progression of symptoms, motor dysfunction becomes relatively stable over long durations of time, except for superimposed periods of accelerated functional decline most often caused by pubertal growth or progressive age-related loss of motor function (Kaufmann et al., 2012). SMA type 2 individuals are considered non-ambulatory and require a motorized wheelchair for independent mobility. Though typically able to self-feed at young ages, most type

2 children lose this skill over time. Furthermore, self-care skills of dressing, bathing, and using restroom facilities (due to transfers to and from the wheelchair) typically require significant if not full assistance. In addition to motor skill impairment, type 2 individuals experience reduced respiratory function due to muscle weakness, though this dysfunction is not as severe as in SMA type 1. Most breathing difficulties arise during sleep or as a consequence of illness, and non-invasive ventilation may be required to reduce pulmonary infections (Schroth, 2009). Despite improved function, respiration difficulties impact life expectancy. Type 2 patients survive into adulthood and have a life expectation of 30 to 50 years of age, contingent upon respiratory function (Bowerman et al., 2017).

1.1.4 SMA type 3

The next classification, type 3, also known as juvenile SMA or Kugelberg-Welander disease, comprises approximately 30% of SMA cases (Kugelberg & Welander, 1956). Unlike the previous categories, type 3 patients are often separated into one of two subsets, dependent upon symptom onset. For children with SMA type 3a, symptoms begin prior to 3 years, while 3b onset is after 3 years. Type 3a children typically achieve but do not keep the ability to walk unassisted, while type 3b retain walking ability throughout life. Proximal and trunk muscle weakness experienced by type 3 patients produces a Trendelenburg gait (a gait abnormality caused by the neuromuscular weakness of hip and lower limb abductor muscles) and hyperlordosis, but these impairments do not significantly reduced quality of life. Children and adults with type 3 have normal lung function and respiratory muscle strength and thus rarely experience respiratory difficulties, with the exception of during illness or as an increased risk for obstructive sleep apnea.

Because respiratory function is not compromised, individuals generally experience normal or near normal lifespans.

1.1.5 SMA type 4

The mildest and rarest form of SMA is type 4, for which symptom onset starts during adulthood (usually after 30 years of age). The percentage of SMA patients with type 4 may be underestimated due to the late onset of symptoms and presence of generalized non-motor symptoms. For example, a recent insurance claim study demonstrated that patients who were initially diagnosed with heart, fertility, or other peripheral abnormalities were later diagnosed with mild SMA (Lipnick et al., 2019). Affected individuals experience progressive but mild proximal weakness with a lower limb predominance, which may result in gait abnormalities and reduced muscle stretch reflexes (Zerres et al., 1995). However, these slowly progressive motor impairments do not affect respiration nor preclude ambulation, and affected individuals do not experience shortened lifespans.

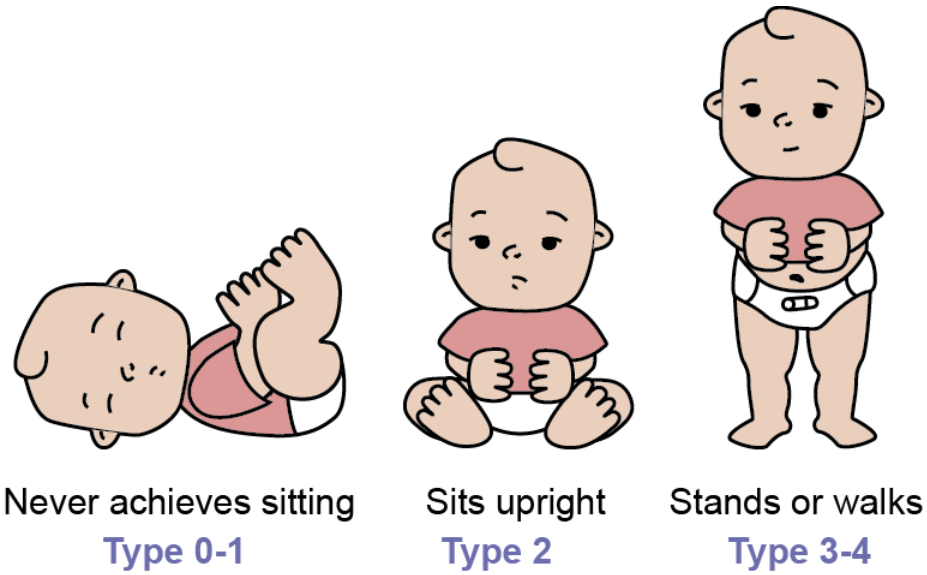

Figure 1 Graphical representation of the traditional classification of SMA types.

SMA patients with types 0 or 1 do not achieve the ability to roll or sit without assistance. Patients with type 2 can sit upright, but never achieve walking. Patients with type 3 or 4 are able to stand or walk (even if briefly).

1.2 The impact of motor impairment on quality of life

SMA patients experience a slow, progressive decline in motor function and report limitations arising from difficulties in mobility, daily activity, and pervasive fatigue associated with deteriorating physical health (Mongiovi et al., 2018; Wan et al., 2020). Though the initial severity of SMA symptoms (and thus the maximal motor function achieved) is variable, depreciation of function results in considerable physical disability for all SMA-affected individuals. Furthermore, inadequate motor function requires daily tasks to be performed by caregivers in order to help maintain or enhance an individual's autonomy. These tasks include activities such as meal preparation or maintaining hygiene, and are usually informally provided by relatives. One Spanish study evaluating the economic burden incurred by SMA patients and their

caregivers found that the invisible "cost" (compared to medical cost) of informal caregiving accounts for more than 2/3 of the total annual cost of healthcare associated with SMA (López-Bastida et al., 2017). This cost is one of the reasons why parents of SMA patients declare that a critical gap in patient needs is the lack of support for activities associated with daily living (Hjorth et al., 2017).

In addition to motor impairment, patients also report emotional difficulties, which thus far lack effective interventions. These difficulties are due in part to the immense psychosocial burdens experienced by those living with SMA. Among the need to make difficult treatment choices, stress, limitations on social activities, and a lack of independence, the pervasive fear of losing functional ability significantly contributes to substantial negative mental health experienced by patients and their families (Qian et al., 2015). When surveyed on which tasks would meaningfully improve quality of life, SMA patients reported that improved ability to do daily tasks such as eating, bathing, grooming, using the restroom and typing on keyboards would be immensely beneficial (Fig. 2). The ability to independently perform these often under-appreciated motor tasks should be a goal for the treatment of SMA, and small changes may be sufficient to significantly improve quality of life. Patient, caregiver and clinician perspectives indicate that even minor improvements in motor function would constitute a meaningful change in disease outcome (McGraw et al., 2017).

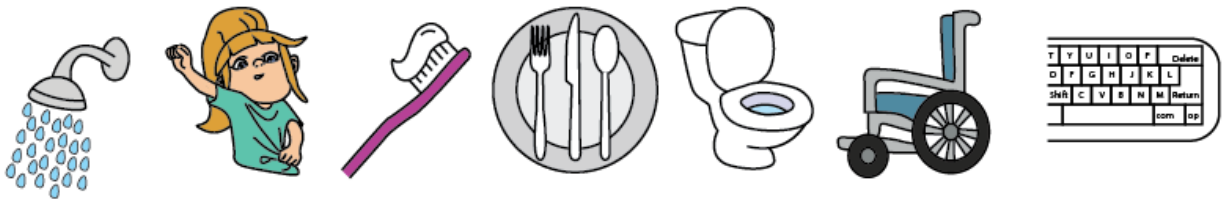

Figure 2 An ability to perform tasks of daily living would provide meaningful improvement to the quality of life experienced by patients and caregivers.

SMA patients and families report that an inability to perform the motor tasks required by daily living is a crucial gap in current care. These tasks require strength, motor skill, and endurance to perform tasks such as transfers

to/from showers, changing clothes, brushing teeth, preparing and/or eating food, transfers to/from facilities or rooms, and typing and/or using a mouse and keyboard.

Until the recent development and use of genetic therapies, management of SMA has traditionally been limited to long-term multi-disciplinary medical, nutritional, and supportive care (Finkel et al., 2018; Mercuri et al., 2018; Mercuri et al., 2020; Wang et al., 2007) to alter the natural disease progression. The advancement of genetic therapies is remarkably reshaping the therapeutic environment by mitigating the need for invasive ventilation and extensive medical care, ameliorating progressive motor degeneration, and improving lifespan. However, key outcomes of clinical trials and therapeutic use indicate that impairments in motor function persist in some patient populations (Finkel, et al., 2017; Mercuri, Darras, et al., 2018; Nurputra et al., 2013; Wadman et al., 2012; Zanetta et al., 2014). Additionally, not all patient populations are able to access or receive genetic therapies. Advances in supportive and therapeutic care have changed the diagnosis of SMA from devastating to hopeful, but it is clear that a true 'cure' for SMA will be achieved through complementary treatment approaches to improve the quality of life experienced by patients and their caregivers.

1.3 The diagnostic journey of SMA

Research in humans and mouse models suggests a therapeutic advantage for diagnosis prior to symptom onset; despite this knowledge, diagnosis of SMA has been historically dependent on clinical manifestation of weakness (Lin, Kalb, & Yeh, 2015). The onset of motor impairment, however, occurs late into disease progression (after substantial motoneuron degeneration) and

motor symptoms can seemingly appear overnight in severe SMA patients. Benefits of therapeutic interventions are significantly limited after significant motoneuron death, and palliative strategies are utilized to maximize symptomatic relief instead of as a method to alter the disease outcome. Substantial diagnostic delays based on symptoms are caused by the difficulty of distinguishing normal from abnormal development, a lack of awareness and knowledge of SMA, and the challenge of differential diagnosis (Qian et al., 2015).

As 95% of SMA cases result from an inherited or random genetic deletion of the Survival of Motor Neuron (*SMN1*) gene, testing for SMA is relatively straightforward. Newborn screening for SMA utilizes the blood spot collected after birth to evaluate *SMN1* deletion. The *SMN1* gene is relatively small, permitting the straightforward assessment of genetic sequence to identify mutations in patients who test negative for deletions. Despite the known importance of early intervention and the ease of testing, newborn screening was not adopted in the United States until 2017 due to a lack of an effective treatment. After the federal approval of an antisense oligonucleotide to treat patients with SMA, Missouri became the first state to institute newborn screening. As of July 1, 2020, screening has been expanded to 31 U.S. States, resulting in the screening of 65% of newborn babies in the United States (Cure SMA, 2020). Newborn screening has been positively received by the SMA community due to shorter diagnostic journeys, an ability to access care sooner, additional time for families to prepare to care for a disabled child, and an improvement in the parent's relationship with a child prior the evidence of symptoms (Qian et al., 2015). With the increasing use of newborn testing for SMA, both within the United States and Europe, patients will have earlier access to remarkable treatments that can radically alter natural disease progression and fundamentally change traditional prognosis.

1.4 Survival of Motor Neuron (SMN) protein

Despite its relative obscurity, SMA is one of the most common diseases of childhood and is the leading genetic cause of death in infants (Prior et al., 2008). The carrier frequency of the genetic mutation that causes SMA is ~1:41, and this disease is typically inherited in an autosomal recessive fashion, with an incidence of SMA of approximately 1:10,000 births worldwide (Pearn, 1978, Sugarman et al., 2012). SMA results from the biallelic loss of the *SMN1* gene, which normally produces adequate levels of a protein called Survival of Motor Neuron (SMN) (Lefebvre et al., 1995). A severe reduction of SMN causes denervation of neuromuscular connections and subsequent degeneration of lower motoneurons generally associated with proximal and axial muscles. Despite the clear origin of this monogenic disease, however, the specific mechanisms underlying pathology remain ambiguous. Why does a paucity of SMN specifically cause motoneuron degeneration? What protective mechanisms are responsible for resistance or vulnerability of particular motoneuron-muscle groups to the degenerative consequences of low SMN?

The last 25 years of research has elucidated crucial insight into disease mechanisms that have been essential for targeting the first class of non-palliative treatments for SMA. Research has resolved the strict temporal requirements for SMN expression, illuminated specific roles of SMN which contribute to cellular and circuitry dysfunction, revealed the complex role of modifiers in phenotypic variability, and realized the peripheral and central loci of disease pathology. The following sections will address outstanding questions and identify critical knowledge gaps necessary to understand in order to develop a cure for SMA.

1.4.1 A tale of two genes: *SMN1* & *SMN2*

Unlike almost all animals, which express a single *SMN* gene, humans are unique in the ability to synthesize SMN protein from two nearly identical homolog genes located within the chromosome loci 5q13 -- telomeric *SMN1* (akin to the non-human *SMN* gene) and centromeric *SMN2* (Lefebvre et al., 1995; Melki et al., 1994; Rochette, Gilbert, & Simard, 2001). Curiously, higher primates are the only known exception to single *SMN* gene expression, though they possess two identical *SMN* copies that efficiently function and thus do not develop SMA if a homozygous *SMN* copy is lost. In humans, the synthesis of functional SMN protein is not equally derived between these two genes -- ~90% of SMN derives from *SMN1* transcripts, and ~10% derives from *SMN2* transcripts.

The *SMN1* gene comprises nine exons and encodes full-length SMN transcripts composed of 294 amino acids with a critical stop codon near the end of exon 7 (Burglen et al., 1996). Only five base pairs differentiate the nearly identical *SMN1* from *SMN2* genes, but a single point mutation in the coding sequence of *SMN2* fundamentally alters the splicing of *SMN2* transcripts. A single cytosine to thymine nucleotide transition at the sixth nucleotide in exon 7 of *SMN2* (Lorson et al., 1999; Monani, McPherson, & Burghes, 1999) does not change the encoded amino acid but does disrupt pre-mRNA splicing by reducing the splice sight recognition capacity of U1 and U2 small nuclear ribonucleoproteins (snRNPs) and other auxiliary factors that recognize the boundary between the exon and intron during pre-mRNA processing (Lorson & Androphy, 2000). As such, about 90% of *SMN2* mRNA transcripts lack exon 7 (Fig. 3), and consequently produces a truncated form of SMN protein composed of only 282 amino acids (Lorson et al., 1999). Additionally, the N-terminus of truncated SMN differs from full-length SMN by the last 4 amino acids and may form a degradation signal (Cho & Dreyfuss, 2010). Mutations in exon 7 disrupt the

capacity for self-oligomerization, which has been demonstrated to be essential for the proper function of SMN (Lorson & Androphy, 1998; Lorson et al., 1998). The reduced stability of truncated SMN results in rapid degradation. While ~100% of SMN derived from *SMN1* transcripts is full length and functional, SMN from *SMN2* transcripts results in only ~10% of full length SMN. For this reason, a reliance on *SMN2* as the sole generator of SMN results in low SMN expression levels.

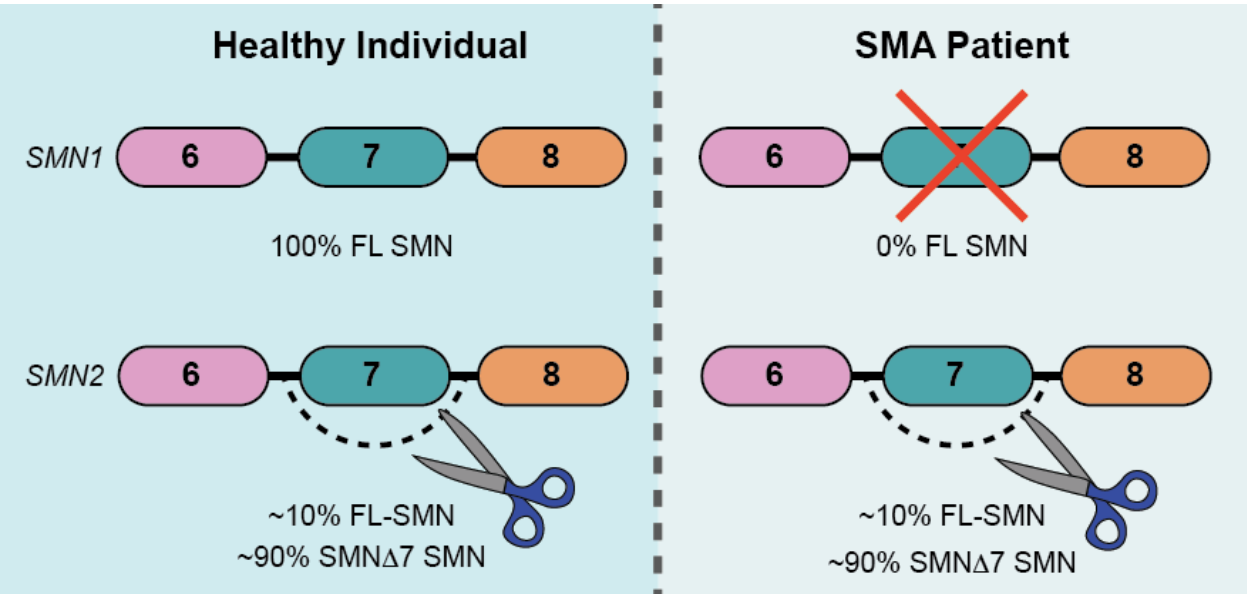

Figure 3 SMA patients rely solely on *SMN2* to produce SMN protein.

Due to a biallelic loss or mutation of *SMN1*, all SMN protein derives from *SMN2* transcripts in patients with SMA. A point mutation in *SMN2* results in ~90% of transcripts lacking a critical exon (exon 7), resulting in protein instability and degradation. FL, full-length; SMNΔ7, SMN lacking exon 7 (truncated).

Mutation in any of the *SMN1* domains has been associated with SMA, though especially mutations in three highly conserved subdomains (Tudor, YG box, and Gemin2-binding regions), indicating that these domains and the overall protein structure are critical for SMN function (Singh et al., 2017). Mutations in *SMN1* are primarily inherited but may arise from de novo mutations (though *SMN1* de novo mutations engender only a small portion of SMA cases). 95% of SMA

cases result from the biallelic deletion of *SMN1*, typically by whole gene deletion but occasionally by gene conversion to *SMN2* (Wirth et al., 1997). These null mutations or deletions consequently cripple the synthesis of full-length SMN.

SMN genes are located in the 5q13 region of the genome (Brzustowicz et al., 1990). Fortunately, this 5q13 region is remarkably amenable to evolutionary lability, and humans can express a variable number of genomic *SMN2* genes (Melki et al., 1994). Higher *SMN2* copy number (and thus higher SMN levels) is inversely correlated with disease outcome (Coovert et al., 1997; Lefebvre et al., 1997). On average, SMA type 1 patients have 2 *SMN2* copies, while type 3 patients have 3-4 copies (Fig. 4). In comparison, non-SMA populations average 1-2 copies, although this average can fluctuate depending on ethnic and genetic background. Thus, SMA is a hypomorphic disease caused by low levels of SMN protein due to the functional loss or deletion of 5q *SMN1*, with retention of variable copy numbers of *SMN2*. To differentiate this disease from other neuromuscular atrophy diseases, such as spinal and bulbar muscular atrophy, SMA is sometimes referred to as 5qSMA or proximal SMA.

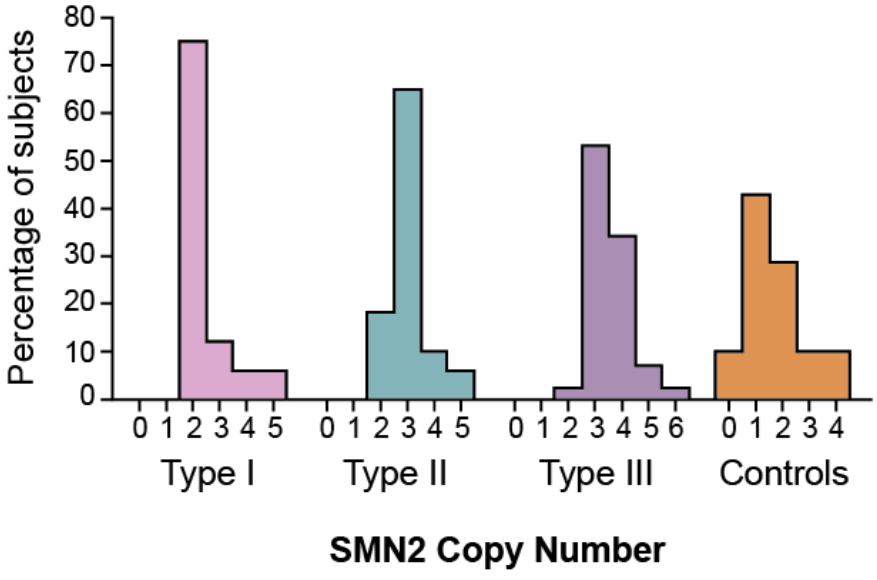

Figure 4 *SMN2* copy numbers in SMA patients and controls.

Control populations have fewer copy numbers of *SMN2* on average compared to SMA patients. Fewer copy numbers correlate with a more severe phenotype of SMA. Figure adapted from (Thomas O. Crawford et al., 2012) doi:10.1371/journal.pone.0033572.g002

1.4.2 Cellular function of the SMN protein

SMN is a 38 kDa protein ubiquitously expressed in eukaryotic cells and is produced from the highly conserved *SMN* gene. As its name suggests, SMN (survival of motor neuron) is an essential protein for the survival of motoneurons, and deleteriously low levels result in lethality during embryogenesis (Schrank, Götz, & Gunnersen, 1997). Full-length human SMN protein contains 294 amino acids within multiple domains, which associate with a variety of binding partners, including Sm proteins, Coilin, Gemin, PR1, and have a capacity to self-oligomerize (Lorson & Androphy, 1998; Singh et al., 2017). The alignment of amino acid sequences is highly conserved for the N-domain, which harbors critical binding sites for interacting partners like Gemin, p53, and nucleic acids (Singh et al., 2017). SMN was canonically thought to locate within nuclear and cytosolic compartments, but immunocytochemical studies have demonstrated that SMN also localizes within dendrites and axons of neurons (Zhang et al., 2007; Zhang et al., 2003). Fluorescently tagged SMN granules have been observed moving bidirectionally and rapidly through neuronal axons via cytoskeletal proteins (Fallini, Bassell, & Rossoll, 2010; Zhang et al., 2003), suggesting a role in axonal transport to neuromuscular synapses.

SMN is a protein that is associated with numerous roles in motoneurons (Fig. 5). At the nucleus, SMN aggregates into protein complexes called ‘gems’ which function to synthesize and correctly assemble snRNPs (small nuclear ribonucleoproteins), which are critical for the proper splicing of messenger RNAs (mRNAs) (Liu & Dreyfuss, 1996). Thus, SMN has a housekeeping

role of regulating pre-mRNA maturation (Pellizzoni et al., 1998). In axons, SMN modulates axiogenesis and axonal pathfinding (Fallini, Bassell, & Rossoll, 2012), and is involved in the axonal transport of RNA and synaptic vesicles (Kye et al., 2014; Rathod et al., 2012). At the synapse, SMN is involved in the local translation of cytoskeletal proteins within presynaptic compartments (Yao et al., 2006). At the growth cone and synaptic terminal of motoneurons, SMN regulates endocytosis and cytoskeleton activity through its interaction with Profilins, a family of proteins regulating actin dynamics (Ackermann et al., 2013; Bowerman et al., 2009; Giesemann et al., 1999; Rossoll et al., 2003). Additionally, SMN regulates the change in G-/F-actin ratio (Bergeijk et al., 2007), which is critical for neurite outgrowth (Fan & Simard, 2002). Augmenting expression of SMN in non-SMA control mice triggers terminal sprouting (although to a limited degree (Rimer et al., 2019)) and suggests that SMN directly influences neuromuscular growth and innervation. After neuromuscular synapses have maturated, SMN contributes to compensatory axonal sprouting following motor nerve injury (Kariya et al., 2014).

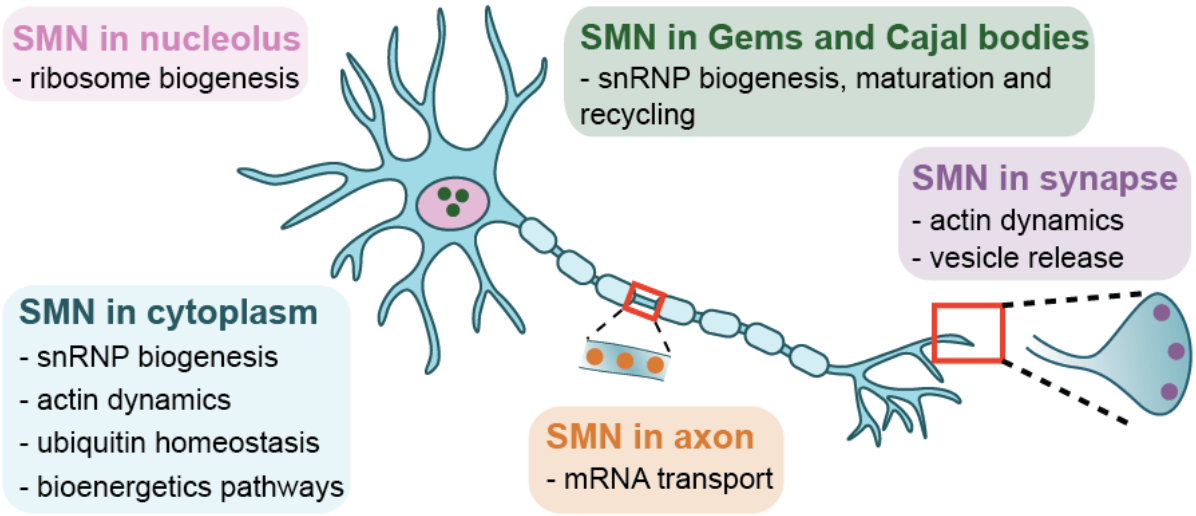

Figure 5 The roles of SMN in the motoneuron.

SMN is associated with several roles in the motoneuron, though which role(s) contributes to specific pathology is unclear. Figure adapted from (Bowerman et al., 2017).

In addition to motoneurons, other cells require sufficient levels of SMN to develop and maturate properly. SMN contributes to the differentiation of satellite cells and myotube formation during fetal stages of muscle development (Boyer, Ferrier, & Kothary, 2013; Hayhurst et al., 2012; Kariya et al., 2014; Shababi, Lorson, & Rudnik-Schöneborn, 2013). In addition to regulating neuromuscular maturation, SMN is required in cells not directly associated with motoneuron circuitry, most notably those involved in cardiac development (Bevan et al., 2010; Heier et al., 2010; Shababi et al., 2012; Shababi et al., 2010). The regulatory roles of SMN in peripheral development are poorly understood, and most of our knowledge has derived from observing organ and cardiac defects in SMN-deficient animal models.

Despite the ubiquitous presence of SMN across a variety of cell types and its pleiotropic roles in cells, the extreme depletion of SMN caused by the loss of *SMN1* is uniquely devastating to lower motoneurons in stages of early maturation and development. Indeed, SMN levels must be precipitously low to cause severe dysfunction. Relative to SMN levels in a healthy population, SMN expression is respectively reduced by ~70%, ~50%, and ~30% in type 1, 2 and 3 patients (Crawford et al., 2012). Low levels of SMN are required by heart and muscle tissues, which are often satisfied by several *SMN2* copy numbers and explain why patients with milder clinical manifestations experience fewer complications with these tissues.

Despite uncertainty in the reason(s) for vulnerability, it is clear that motoneurons exhibit the most severe pathology as a consequence of low SMN. Peripheral SMN expression is required to improve lifespan and pathology in severe SMA model mice (Gavrilina et al., 2008; Hua et al., 2015), and restoration of SMN in motoneurons is necessary to improve function and mitigate pathology (Gogliotti et al., 2012; Kariya et al., 2014; Laird et al., 2016; Lee et al., 2012; Park et

al., 2010). SMN functions at presynaptic motor terminals cell-autonomously to bolster synaptic transmission (Martinez et al., 2012), which is important to note since neuromuscular development is regulated by synaptic activity. Thus, the role of SMN in developing motoneurons, and particularly its role in peripheral function and maturation, is clearly critical. The mechanism(s) underlying the selective deterioration of motoneurons remains elusive, but one possibility is that the aberrant orchestration of snRNP biogenesis or defective splicing experienced by motoneurons lacking SMN results in aberrant or unsustainable synaptic connections. Indeed, defects in neuromuscular transmission, motoneuron hyperexcitability, and reductions of synaptic contacts onto motoneuron cell bodies (Gogliotti et al., 2012; Ling et al., 2012; Mentis et al., 2011) are characteristic features of SMA pathology. However, the roles of SMN in axonal transport or nerve terminal translation, independent of its roles in RNA metabolism, may also contribute to neuromuscular dysfunction. A detailed evaluation of pathology caused by SMN deficiency can be found in section 1.6.

1.4.3 Temporal requirements of SMN during development

In humans and mammals, SMN expression levels are highest during embryonic and early postnatal development, followed sharply by a decrease to a basal level that is maintained throughout life (Gabanella et al., 2005; Kariya et al., 2014). However, the complex mechanisms regulating dynamic expression are not well understood. Peak SMN protein levels in the spinal cord are the highest during the developmental window of axon sprouting from the spinal cord during early embryogenesis (Burlet et al., 1998; Jablonka et al., 2000) and coincide with the onset of myelination (Gabanella et al., 2005). In mice, maximal SMN expression occurs during embryonic day 10-13 to permit the growth and pathfinding of motoneuron growth cones to contact target

muscles (Jablonka et al., 2000). Motoneuron cultures isolated at E13.5 from SMA mouse model spinal cords do not display accelerated death, indicating that SMN expression derived from *SMN2* is sufficient to prevent lethality (Rossoll et al., 2003). However, cultured growth cones are smaller, exhibit shorter axons, have lower β-actin expression and reduced calcium transients (Jablonka et al., 2007; Rossoll et al., 2003). SMA patient-derived induced pluripotent stem cell motoneurons and SMA model morpholino zebrafish display axon elongation and pathfinding deficits, but these defects were not found in mouse models or patient tissue (Kariya et al., 2014; McGovern et al., 2008). This evidence suggests that severe defects caused by SMN deficiency occur at late states of differentiation instead of early stem cell or neuronal development stages.

In mice, SMN undergoes an initial decline in expression between embryonic days 14 and 19, after the developmental period of motoneuron innervation of muscle endplates (Ling et al., 2012). Increasing neuronal SMN (but not muscle SMN) at embryonic day 15 corrects the SMA phenotype seen in SMA mice (Gavrilina et al., 2008), further suggesting that the perinatal development period requires elevated neuronal SMN expression. A secondary decline occurs between postnatal day 5 and 15 (Fig. 6) (Jablonka & Sendtner, 2017), which occurs when neuromuscular junctions begin to stabilize and mature. Early postnatal restoration of SMN in a severe mouse model rescues neuromuscular pathology and muscle weakness, indicating the critical requirements of SMN expression during the time frame of neuromuscular maturation (Lutz et al., 2011).

The switch from SMN requirement to insensitivity occurs abruptly at P17 in SMNΔ7 mice (a popular mouse model of severe to intermediate SMA; see section 1.5), which coincides with the end of presynaptic maturation (Kariya et al., 2014; McGovern et al., 2008). This timing also coincides with a relative decline in the activity of SMN in assembling snRNP particles in mouse

spinal cord tissue (Pellizzoni, 2007). By postnatal day 20, low levels of SMN, which can be satisfied by two copies of *SMN2* in a mouse model, adequately maintain mature neuromuscular synapses (Kariya et al., 2014). In fact, knock-down of SMN in adult mice has relatively minimal consequences, except for reduced regenerative capability in response to nerve injury (Kariya et al., 2014). Therefore, SMN is required for neuromuscular maturation, as well as regeneration or repair pathways later in life. The need for SMN to maintain neuromuscular integrity suggests that curative therapies should be delivered during the critical stages of neuromuscular maturation in order to prevent the establishment of irreversible defects that cause lifelong neuromuscular impairment (Fig. 6).

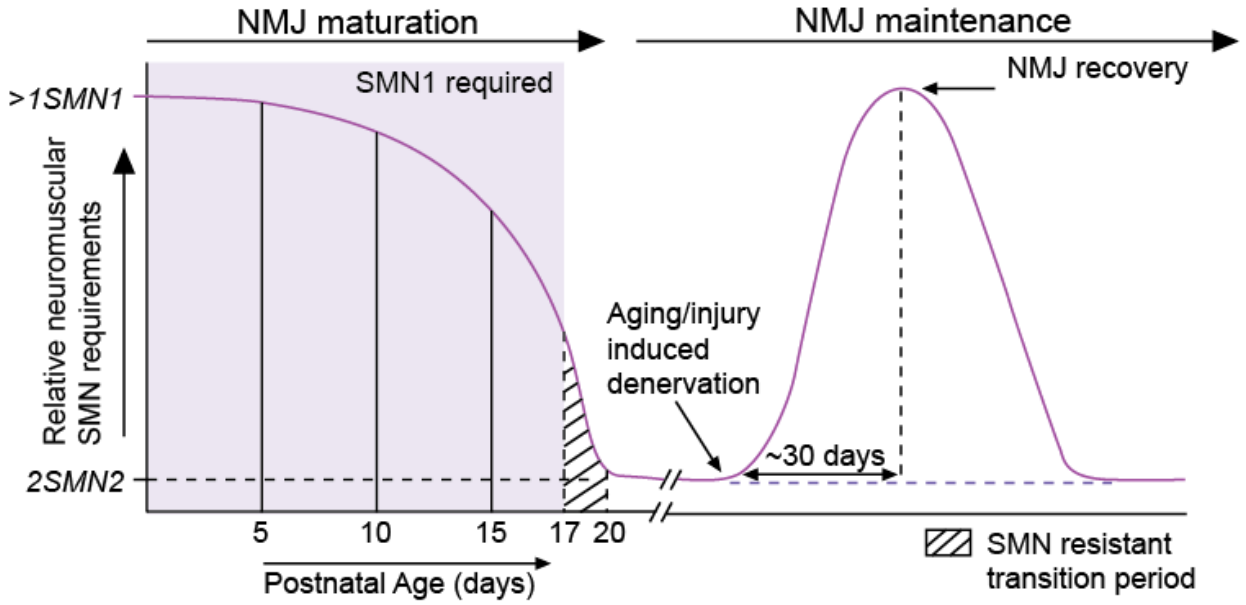

Figure 6 Temporal requirement of SMN expression in mice.

SMN expression is highest during the perinatal period (purple highlight), but precipitously drops after neuromuscular development (around 17-20 days of age; striped highlight), when NMJs become viable with low levels of SMN (satisfied by 2 copies of *SMN2*). Remodeling due to aging or injury requires elevated SMN levels to recover and stabilize NMJs. Figure adapted from (Kariya et al., 2014).

1.5 Animal models versus humans: Cautious interpretation of disease mechanisms

The SMN gene has a high degree of evolutionary conservation throughout the animal kingdom, and several SMN orthologs have been identified, from fungi, fruit flies, yeast, honeybees, oysters, chickens, worms and mice, which have permitted the development of a variety of animal models of SMA (Schmid & DiDonato, 2007). Preliminary experiments knocking out the *SMN1*-equivalent gene in model organisms established that the consequence of full SMN loss is embryonic lethality (Chan et al., 2003; McWhorter et al., 2003; Miguel-Aliaga et al., 1999; Schrank et al., 1997). Since only humans have a homolog gene (*SMN2*) that can partially compensate for the loss of *SMN1*, SMA is a uniquely human disease. Therefore, genetic manipulation of animal models has been necessary to study SMA pathogenesis and to evaluate preclinical treatments. Faithful replication of this human-specific disease has been demonstrably complex, however. Intrinsically dynamic SMN expression during development, the presence of maternally derived SMN in some animal models, and the timing of SMN knockdowns make extrapolation of results to the human disease complicated.

Despite these considerations, genetically manipulated animal models have become pivotal for elucidating the mechanisms and etiology of disease processes and have permitted the extensive research necessary to thoroughly screen and test potential treatments and cures. Major advances in understanding the neuromuscular and sensory pathology caused by SMA and temporal requirements for treatment intervention have largely been achieved through genetically manipulated animal models. Additionally, as SMN is involved in a variety of roles in the cell, individual models have provided unique information about the function of SMN within specific cell populations and the consequence of its reduction or absence.

For example, drosophila melanogaster (fruit fly) SMA models exhibit defective

locomotion, neuromuscular transmission impairment (Imlach et al., 2012) and neuromuscular pathophysiology (Chan et al., 2003; Imlach et al., 2012; Praveen et al., 2014; Praveen, Wen, & Matera, 2012), which coincide with findings in other SMA animal models and humans. However, the precise neuromuscular pathology does not align with what is seen in vertebrates; pathology consists of poorly aligned pre- and post-synaptic contacts (Chan et al., 2003) and mislocalized glutamate receptors on muscle cells, which are not reflected in mammalian models or SMA patient tissue. These differences are likely due, in part, to the high levels of maternal SMN transcripts and proteins expressed during the initial half of the larvae stage, which does not decline until division into daughter cells. However, fruit flies have provided a distinctive opportunity for the assessment of SMA patient-derived point mutations. Praveen et al report a broad range of phenotypic severity in fruit flies modeled via patient-specific mutations, which recapitulate the human disease (Praveen et al., 2014). Research in flies has permitted the rapid and low-cost observation of SMA-causing mutations that result in neuromuscular dysfunction without significant disruption of spliceosomal RNP assembly.

Danio rerio (zebrafish) has also been an excellent model for understanding SMN function and SMA pathology. This model serves as an in-between of mammals and invertebrates. Models have been created using morpholinos, which are modified antisense oligonucleotides that transiently suppress endogenous protein expression or alter splicing patterns upon injection into zebrafish embryos (McWhorter et al., 2003). Despite the morpholino degradation over time (thus decreasing its effectiveness in protein down-regulation), titration of the antisense oligonucleotide permits dose-dependent decreases of protein levels at critical time points during development. SMN that is specifically decreased in motoneurons during early development to levels similar to those found in patients (Nasevicius & Ekker, 2000) results in cell-autonomous defects without

affecting muscle development, indicating that a motoneuron-specific deficiency is sufficient to cause pathology similar to SMA. Zebrafish have also been helpful in understanding modifiers of human SMA phenotypes found in some discordant families (which are families composed of two or more children with identical copy numbers of *SMN2* yet have distinct phenotypes) (Oprea et al., 2008). One of these modifiers, Plastin 3, is a calcium-regulated actin-binding protein that is regulated by SMN that can modify neuromuscular pathology when overexpressed in SMA zebrafish models (Hao et al., 2012). Zebrafish models have also permitted the investigation of other modifiers, such as stasimon (Lotti et al., 2012), chondrolectin (Bäumer et al. 2009; Zhong et al., 2012), and neurexin2a (See et al., 2014) which have all also been found to be irregularly spliced or expressed in SMA mouse models. The ability to analyze the transcriptome of SMN morphant embryos revealed that these modifiers are mis-spliced due to low SMN levels and confirms a cross-species effect of SMN deficiency.

The most influential SMA animal model, however, has been mus musculus (house mouse). This model has indelibly helped researchers to understand disease mechanisms and etiology. This model has also been integral for identifying and confirming novel therapies, several of which have either achieved or are in the process of achieving FDA-approval for treatment of patients. A variety of mouse models have been created to reflect the range of clinical phenotypes, permitting targeted evaluation of treatments that may optimally benefit one classification of SMA patient. Critically, most of the elements involved with regulating pre-mRNA splicing of the *SMN* gene are highly conserved in mice (DiDonato et al., 2001). Transgenic induction of the human *SMN2* gene into mice with a disrupted *SMN* gene resulted in the first viable SMA mouse model. Pups that were homozygous for *SMN* disruption while expressing human *SMN2* were able to survive embryogenesis but display severe hind limb weakness and death at 5 days of age (Hsieh-Li et al.,

2000; Monani et al., 2000; Schrank et al., 1997). However, this rapid loss of function and critically shortened lifespan -- representative of the most severe form of SMA -- limited the experimental window for evaluating etiology or testing potential treatments. In the decades since these studies, dozens of mouse models have been created. A variety of genetic techniques have been utilized to develop novel mouse lines, including inducible SMN lines, tissue-specific *SMN* gene knockouts, and highly specific genetic knockouts to recapitulate severe, intermediate or mild SMA phenotypes or patient-derived missense mutations. The most commonly used mouse model is the SMNΔ7 mouse (colloquially the "delta 7 mouse"). Similar to the initial mouse model described above, the SMNΔ7 model expresses a null *SMN* background with a human *SMN2* transgene, but also expresses an additional transgene, *SMN2*Δ7. *SMN2Δ7* lacks exon 7, but mildly improves the SMA phenotype in mice. These triple transgenic mice have approximately double the lifespan (11 days), and display severe motor impairment, neuromuscular pathology, and motoneuron loss (Le et al., 2005). SMNΔ7 represent a phenotype between severe and intermediate. For these reasons, the SMNΔ7 model has been extensively used to understand disease mechanisms as well as test preclinical therapeutics.

1.6 Cellular and molecular spinal cord pathology caused by SMN deficiency

Due to its role in spliceosomal machinery and RNA metabolism, deficits caused by extreme SMN deficiency can be measured in a variety of cell types, and full SMN depletion is not compatible with life for any cell. However, few tissues are as exceptionally affected by low levels of SMN as lower α-motoneurons. The pathology of SMA is notable for the severe, characteristic abnormalities of motoneurons, axons, and neuromuscular synapses. Does SMN have a

motoneuron-specific role that could explain the exceptional susceptibility to somata degeneration? Mouse models have shown that specific SMN restoration in motoneurons corrects pathologies and phenotypes associated with SMA (Gogliotti et al., 2012) but does not prevent premature death. However, systemic restoration of SMN is necessary to dramatically improve lifespan of severe SMA mice, suggesting that cells other than motoneurons contribute to the pathogenesis of SMA (Hua et al., 2011). One possibility is that lower α-motoneurons require highly elevated levels of SMN during development, while other cells, such as proprioceptive sensory afferents and skeletal and cardiac muscle cells, require lower levels of SMN. In support of this possibility, significantly more structural cardiac defects have been diagnosed in SMA type 1 patients than in SMA type 3 patients, who exhibit milder defects like rhythmic abnormalities (Shababi et al., 2013; Wijngaarde et al., 2017). While it is clear that motoneurons are deleteriously vulnerable to low levels of SMN, other cells may experience defects as well. This section will describe the primary and secondary pathophysiologies caused by SMN deficiency in motoneuron and non-motoneuron cells.

1.6.1 Pathology of cells in the spinal cord

The most obvious pathology upon autopsy is the swelling and degeneration of motoneurons in the anterior horn of the spinal cord. This degeneration is driven by the induction of the p53 signaling pathway. p53 upregulation occurs in pre-symptomatic states of vulnerable motoneurons in SMA mice (Simon et al., 2017). Curiously, the nuclear accumulation of p53 is observed in resistant motoneurons during late disease stages but is not associated with cell death. While the mechanism(s) regulating the p53-driven motoneuron degeneration are poorly understood, tissue from humans and animal models have demonstrated that degeneration of motor somata is relatively restricted; only a small number of motoneurons experience degeneration prior to the onset of death

in humans and mouse models. Severe degeneration is often localized in the medial column of upper lumbar segments (L1-3), which primarily correspond to proximal muscle groups (Mentis et al., 2011), while motoneurons innervating distal limbs within L4-5 are more resistant to denervation. This evidence suggests that pathophysiology occurring prior to degeneration significantly contributes to phenotypic dysfunction. Animal models of SMA have shown that prior to degeneration, motoneurons exhibit a loss of proprioceptive input (Mentis et al., 2011). Proprioceptive afferents sense the position of muscles in relation to the trunk (Lam & Pearson, 2002; Windhorst, 2007) and contribute significant afferent input to modulate motoneuron firing to regulate muscle tone, which is critical for postural control (Davidoff, 1992; Dietz & Sinkjaer, 2007). Is the loss of synaptic input due to motoneuron SMN deficiency (motoneuron-autonomous) or a consequence of SMN loss in other connected cells (interneurons, sensory neurons, astrocytes and microglia)? The answer to this question is not clear, but it is evident that SMN in motoneurons is critical for circuitry in the spinal cord. Depletion of SMN in motoneurons causes central and peripheral synaptic stripping (McGovern et al., 2015; Park et al., 2010), and selective restoration of SMN in motoneurons improves motor function and synaptic circuitry (Arnold et al., 2016; Gogliotti et al., 2012; Martinez et al., 2012). Additionally, administration of Trichostatin A, a histone deacetylase inhibitor that increases *SMN2* transcription, can reverse proprioceptive stripping (Avila et al., 2007).

Another pathophysiological feature of SMN deficient motoneurons is a change in intrinsic excitability, which precedes motoneuron loss (Fletcher et al., 2017; Mentis et al., 2011; Quinlan et al., 2019). While it is understood that motoneurons exhibit hyperexcitability, the etiological basis of this aberrant functioning is debated. SMN deficient cultured motoneurons with complex synaptic networks and SMA mouse models show decreased action potential thresholds and a larger

density of voltage-dependent sodium currents (Arumugam et al., 2017; Quinlan et al., 2019). These results were also observed in singular NCS-34 motoneuron-like cells lacking synaptic inputs (Arumugam et al., 2017). However, motoneurons from an SMA mouse model show that proprioceptive afferents regulate the surface expression of the voltage-gated potassium channel Kv2.1 on motoneurons, which modulates motoneuron excitability (Fletcher et al., 2017).

Aside from motor and sensory neurons, other spinal cord cells may influence disease pathology. Selective SMN deficiency in interneurons is sufficient to reduce synaptic contacts onto wildtype motoneurons (Simon et al., 2016). SMN deficiency also causes astrogliosis in spinal cords of end-stage SMA mice and patients (Rindt et al., 2015). SMA mouse models have reduced astrocyte-motoneuron coupling during development, which could alter the maintenance of synaptic contacts within the spinal cord (Rindt et al., 2015; Zhou, Feng, & Ko, 2016). Abnormal calcium regulation and reduced growth factor production has also been observed in SMN-deficient astrocytes (McGivern et al., 2013), and viral-based restoration of SMN to astrocytes improves neuromuscular circuitry and overall lifespan (Rindt et al., 2015). Intrinsic defects have also been observed in myelinating Schwann cells, including failure to respond to myelination cues in cultured cells and myelination defects in SMA mice (Hunter et al., 2014; Sarvestany et al., 2014), which can be reversed with Schwann cell-specific restoration of SMN (Hunter et al., 2016). These data suggest that non-neuronal cells may contribute to SMA pathogenesis.

Aberrations in spinal cord circuitry caused by SMN deficiency can negatively affect the maintenance of synaptic connections. Developing circuits are shaped by synaptic activity, and changes in activity can reinforce highly active synapses or remove weak synapses to control the maturation of a circuit. This plasticity is retained in mature synapses, where potentiation or depression of activity can alter the ability of a postsynaptic cell to initiate an action potential.

Further investigation is needed to understand the role of SMN in regulating circuitry and function in cells of the spinal cord.

1.6.2 Neuromuscular development in the presence and absence of adequate SMN expression

Investigations of the temporal requirements of SMN have determined that SMN is an essential regulator of motoneuron development during the period of neuromuscular maturation. Deleteriously low SMN levels in humans and mouse models result in maturational delays, decreased neuromuscular transmission, and dissolution of synaptic contacts in highly vulnerable muscles. Neuromuscular pathology is one of the earliest detectable consequences of SMN deficiency (Doktor et al., 2017; Ling et al., 2012), and precedes motoneuron degeneration. The dysfunction of neuromuscular synapses is a critical component of SMA pathogenesis, and a key therapeutic target for improving motor function (Boido & Vercelli, 2016; Bowerman et al., 2017).

1.6.2.1 Neuromuscular development is regulated by synaptic activity

Neuromuscular junctions (NMJs) are specialized tripartite synapses that transmit information from the central nervous system to control movement by triggering the firing of muscle fiber action potentials. The initial differentiation of motoneurons and muscles occurs independently until initial contact between them is established (Arber, Burden, & Harris, 2002). Multiple motoneurons synapse onto a single myofiber endplate (composed of acetylcholine receptors and associated binding partners), which requires synaptic pruning (also called “synapse elimination”) to achieve mono-innervation by the end of the maturational period. Motoneuron growth cones contain functional transmitter release mechanisms prior to synaptic contact, and each

presynaptic terminal can elicit effective neuromuscular transmission upon endplate contact (Sanes & Lichtman, 1999; Slater, 2017).

After establishment, the maturation and pruning of NMJs is regulated by synaptic activity and neurotrophic factors. Pruning is regulated by Hebbian potentiation and synaptic competition for the muscle-derived pro-peptide BDNF (brain-derived neurotrophic factor; pro-BDNF) (Lu, 2003; Park & Poo, 2013; Poo, 2001; Schinder & Poo, 2000). Pro-BDNF is developmentally regulated, with the highest expression in mice during the first two weeks of development (Yang, Je, Ji, & Nagappan, 2009). Transcription of pro-BDNF is calcium-dependent and tightly regulated by presynaptic activity (Yang et al., 2009). Once pro-BDNF is secreted into the extracellular basal lamina, proteolytic cleavage of pro-BDNF is regulated by matrix metalloproteinases (MMPs) (Garcia et al., 2010; Shilts & Broadie, 2017). Nerve secretion of MMPs is also activity-dependent and converts pro-BDNF into mature BDNF (mBDNF), which binds to the tyrosine kinase receptor TrkB to nurture synaptic contact. In contrast, uncleaved pro-BDNF promotes synaptic depression and retraction by binding to the pan-neurotrophin receptor p75NTR (Je et al., 2013; Je et al., 2012; Singhal & Martin, 2011).

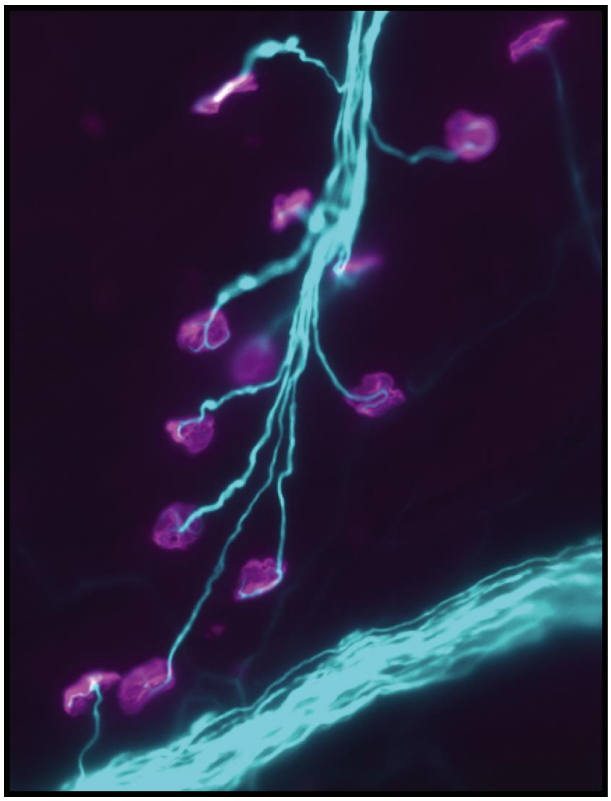

Figure 7 Mono-innervation of neuromuscular junctions during maturation.

Endplates of postnatal day 12 wildtype mouse epitrochleoanconeus muscles are innervated by single axons (cyan, neurofilament; magenta; α-bungarotoxin).

Presynaptic development includes the growth of nerve terminals, accumulation of acetylcholine-containing synaptic vesicles, increase in the number of synaptic vesicle release sites, and a refinement of cytoarchitecture arborization (via radial growth and elongation) to precisely juxtapose postsynaptic receptor clusters. As the synapse matures, nerve terminals increase the magnitude of transmitter release, also known as quantal content (Wood & Slater, 2001). Nerve terminals with the greatest quantal content, in comparison to competing nerve terminals, "win" the competition to singly innervate a postsynaptic endplate while all other terminals retract (Fig. 7). In parallel, endplates evolve from small, plaque-like structures with a congruent density of acetylcholine receptors to large and intricate "pretzel-like" structures punctuated by distinct

clusters of acetylcholine receptors (Fig. 8) (Matthews-Bellinger & Salpeter, 1983). Acetylcholine receptors also undergo maturational changes by increasing in density and switching their subunit composition from including the fetal γ subunit to the adult ε subunit, in addition to the constant presence of two α, one β, and one δ subunit in this pentameric channel (Albuquerque et al., 2009).

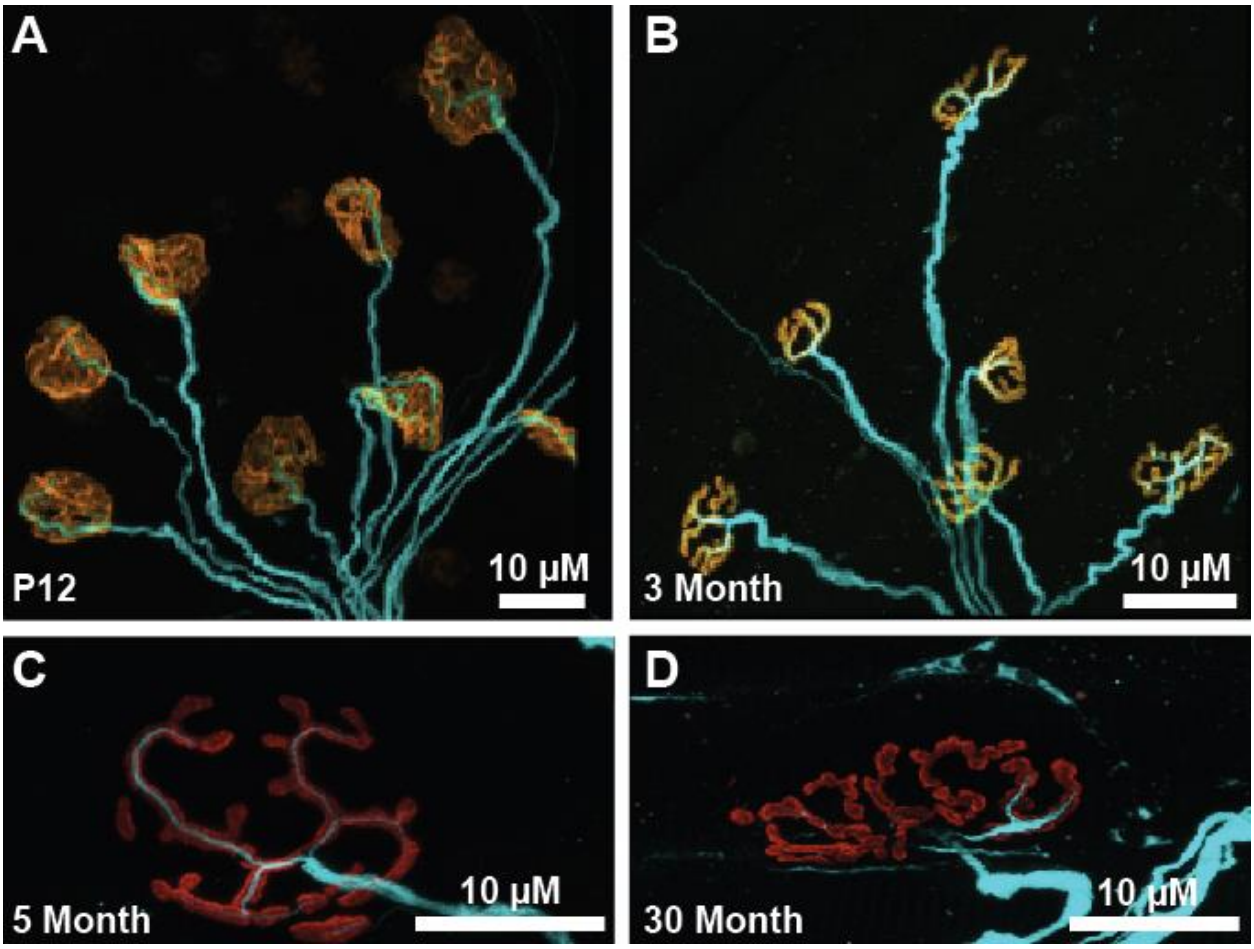

Figure 8 The structural reorganization of muscle fiber endplates through life.

(A) During perinatal development, endplates appear as dense clusters of acetylcholine receptors, which become punctuated by perforations as the synapse maturates. (B) After NMJ development, endplates are characterized as "pretzel"-like shapes, with distinct and intricate branches. (C) Endplates continue to expand radially during adulthood and develop 'islands'. (D) Endplates are predominantly composed of distinct islands. Cyan, anti-neurofilament; orange/red, α-bungarotoxin (to label acetylcholine receptors). Micrograph credit for (C-D): Nick Li.

Several molecules regulate the gradual evolution of postsynaptic organization. The best characterized molecule is the proteoglycan agrin, which is expressed presynaptically in the motoneurons as z^+ agrin (Bolliger et al., 2010). Z^+ agrin promotes the clustering of acetylcholine receptors by binding to Lrp4, which subsequently induces the phosphorylation of the muscle-specific receptor tyrosine kinase, MuSK (Gautam et al., 1996) (DeChiara et al., 1996; Jennings & Burden, 1993). MuSK interacts with Dok-7, a muscle-expressed protein that is also necessary to induce AChR clustering (Okada et al., 2006). Several other molecules contribute to postsynaptic maturation, including the WNT signaling pathway (Korkut & Budnik, 2009), trophic factors (Umemori et al., 2004), neuregulins (Jo et al., 1995), (Jaworski & Burden, 2006; Sandrock et al., 1997), and extracellular matrix proteins such as laminin (Fox et al., 2007; Noakes et al. 1995; Singhal & Martin, 2011). As the endplate matures, an increasingly focused accumulation of Nav1 channels is expressed within the growing postsynaptic folds (Bailey et al., 2003). The high concentration of Nav1 channels mediate explosive depolarization after initiation by the opening of acetylcholine receptor channels, which triggers the muscle action potential (Flucher & Daniels, 1989).

In addition to nerve and muscle contributions to neuromuscular development, perisynaptic Schwann cells also play an integral role in the development and organization of NMJs (Alvarez-Suarez, Gawor, & Prószyński, 2020). While not necessary for the initial stages of NMJ formation, these non-myelinating Schwann cells cap the motor nerve endings and promote synaptic growth, maturation and maintenance during development (Ko & Robitaille, 2015). The molecular mechanisms of how Schwann cells regulate synaptic growth and maintenance are not well understood. One potential role of these cells is to aid in the removal of excess nerve terminals at

multiply innervated NMJs via phagocytosis of retracting terminals (Bishop et al., 2004; Griffin & Thompson, 2008). However, Schwann cells do not appear to select the winning nerve terminal among competing synapses (Smith et al., 2013).

In humans, the initial contact between motoneurons and a muscle fiber coincides with 8-10 weeks of gestational age, and the pruning of superfluous axons occurs prior to birth (MacIntosh, Gardiner, & McComas, 2006). In comparison, mono-innervation is achieved by the second week after birth in mice (Sanes & Lichtman, 1999), and neuromuscular endplate maturation is completed around postnatal day 21. Humans have the smallest studied vertebrate NMJ and these very small NMJs correspond with exceptionally extensive postsynaptic folds that contain high concentrations of voltage-gated Na^+ channels. The spacing and depth of these folds allows for effective amplification of transmitter action, thus permitting the use of low quantal content to excite the postsynaptic fiber (Slater, 2017). However, low quantal content also causes human NMJs to be exceptional vulnerable to disease processes that impair release (Hughes, Kusner, & Kaminski, 2006). In normal conditions, a mature NMJ releases more transmitter than necessary to excite the muscle fiber, a feature known as the "safety factor". The safety factor ensures reliable transmission even during the most extreme voluntary exertion (Wood & Slater, 2001). In humans, the safety factor in response to low frequency stimulation is about 2 (meaning the nerve releases about double the amount of transmitter required to initiate a postsynaptic action potential), and is likely lower during intense activity (Slater, 2017). In comparison, the safety factor of rodents is about 4, so even a 50% reduction in transmission would still reliably trigger postsynaptic muscle action potentials.

Synaptic activity is clearly a critical component of neuromuscular development. Transmitter release drives pre- and postsynaptic maturation, mediates synaptic pruning, and is

necessary to induce fiber contraction in response to a variety of physiological stressors. Therefore, neuromuscular transmission must be consistently reliable. What mechanisms regulate synaptic transmission at neuromuscular junctions?

Transmitter release at synapses is triggered by calcium entry into nerve terminals. A small fraction of presynaptic voltage-gated calcium channels (VGCCs) open in response to the depolarization caused by the brief action potential invasion of the nerve terminal (Brenowitz & Regehr, 2007; Dittrich, Homan, & Meriney, 2018; Luo et al., 2011; Tarr, Dittrich, & Meriney, 2013; Wachman et al., 2004). These channels are thought to lie within an ordered array of intramembranous particles that rigidly align with readily releasable synaptic vesicles (Nagwaney et al., 2009). These sites (called 'active zones') are tightly juxtaposed to postsynaptic folds and are the location of vesicle fusion in nerve terminals (Figure 9). Vesicles are axonally transported from the soma via actin cytoskeletons to active zones, where they are docked to the membrane by the SNARE protein complex. Calcium triggers vesicle fusion upon binding to synaptotagmin (syt1/2 at NMJs), a calcium-sensitive release sensor imbedded within vesicle membranes that is tightly associated with the SNARE complex. Upon vesicle fusion, acetylcholine (the primary transmitter at vertebrate NMJs) diffuses across the narrow synaptic cleft to bind to postsynaptic acetylcholine receptors. Binding of acetylcholine causes these receptor channels to open, inducing depolarization and generating an action potential to trigger muscle contraction (Boyd & Martin, 1956; Katz & Miledi, 1967, 1979). Calcium entry into active zones is not normally large enough to reliably trigger transmitter release at all active zones (Tarr et al., 2013); thus, calcium elevation only initiates transmitter release at a subset of active zones. NMJs derive strength and reliability by the assembly of hundreds to thousands of these low probability active zones within the NMJ (Laghaei et al., 2018). Therefore, transmitter release at single active zones is a low probability event in

normal conditions, and any factors that reduce calcium entry may severely disrupt synaptic activity.

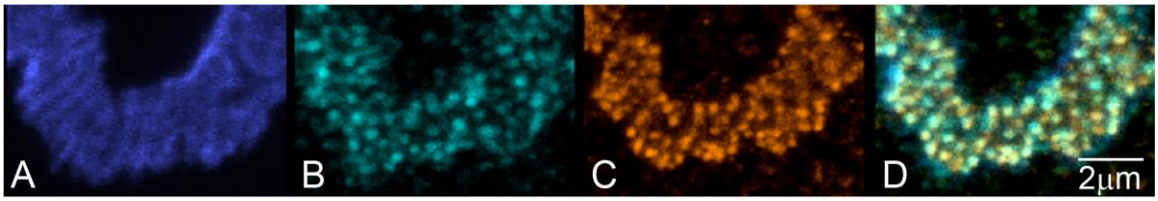

Figure 9 Synaptic vesicle release sites at neuromuscular junctions.

Micrograph of a small portion of a mouse NMJ. (A) Postsynpatic endplates (as visualized via α-bungarotoxin labeled acetylcholine receptors). (B) Immunohistochemically labeled P/Q voltage gated calcium channels can be seen as distinct puncta. (C) Active zones are labeled by using antibodies directed against bassoon proteins. (D) Overlay of A-C shows close alignment of active zones and calcium channels over endplates.

A single motoneuron and all of the skeletal muscle fibers it innervates is known as a "motor unit". When a motoneuron fires an action potential, all of its associated NMJs should release sufficient acetylcholine to excite each muscle fiber and induce contraction. Force of contraction is controlled by the number of motoneurons (thus number of fibers) participating in the movement. Therefore, motoneuron recruitment is an essential regulator of voluntary movement, and impairment of mechanisms that lead to this recruitment, as well as impaired neuromuscular transmission, can have devastating consequences to motor function (Hodson-Tole & Wakeling, 2009).

1.6.2.2 Neuromuscular pathology caused by SMN deficiency

Evidence suggests that SMN is essential for the normal maturation of motor nerve terminals (Torres-Benito et al, 2011), the ability to regenerate in response to damage, and for the resistance to degeneration caused by normal aging. SMN deficiency during neuromuscular maturation alters the development of some or all of the tripartite cells, leading the NMJ to be

selectively vulnerable to subsequent degeneration over time. As neuromuscular symptoms are some of the first detectable defects, early therapeutic interventions (prior to the onset of clinical symptoms) are necessary to decrease the likelihood of irreparable damage and the prospect to halt the disease progression, mitigate muscle atrophy, and improve motor function.

Animal models and patient tissue has provided powerful and compelling evidence that α-motoneurons and their neuromuscular synapses are an early pathological target in SMA. In particular, NMJ defects are observed prior to overt clinical symptoms, and much of the pathology begins during the middle of perinatal maturation (the first two weeks in mice). Additionally, NMJ denervation is apparent prior to the degeneration of cell bodies. However, due to the relative rarity of patient tissue for analysis, much of our understanding of pathogenesis has been driven through research on animal models. In particular, the SMNΔ7 mouse model has been integral for evaluating the dramatic abnormalities in NMJ morphology. Three main anatomical NMJ pathologies are starkly evident in patient and mouse models, including neuromuscular immaturity, denervation, and neurofilament accumulation (Figure 10) (Boido & Vercelli, 2016; Cifuentes-Diaz, Nicole, & Velasco, 2002; Kariya et al., 2008; Ling et al., 2012). Curiously, NMJs within a single muscle show variability in the degree of this pathology in both patients and mouse models, with some NMJs spared of defects while others are consumed.

A key component of this pathology is the widespread breakdown and loss of NMJs (leading to denervation). Curiously, and for mechanisms unknown, denervation is restricted to some muscles (deemed "vulnerable"). Several groups have reported the presence of retracted growth cones or vacant endplates. Partial occupancy of presynaptic terminals over endplates indicate a process of "dying back" axonopathy caused by the retraction of motor terminals from its target muscle fiber due the degeneration of presynaptic terminals (Murray et al., 2008). Denervation

underlies some of the paralytic clinical symptoms experienced by SMA patients (with subsequent motoneuron death also contributing). Functional loss of singular NMJs or entire motor units result in progressively weaker compound muscle action potentials over time, as motoneuron degeneration is irreversible. In milder forms of SMA, compensatory sprouting can mitigate consequences of motoneuron degeneration (Kuru et al., 2008; Ruiz & Tabares, 2014). Curiously, 50% of endplates are denervated prior to the onset of muscle weakness in the SMNΔ7 mouse (Ling et al., 2012).

The extent of denervation is variable but primarily distinguishable by muscle group. Severe denervation (>50%) appears predominantly in vulnerable axial and appendicular muscles (Ling et al., 2012), while other muscles are mildly or entirely resistant to denervation at end stages of the disease in SMA model mice. Curiously, two muscles involved in respiration display opposing vulnerability: intercostal muscles are highly susceptible to atrophy and denervation in humans and mouse models, while the diaphragm is spared or even stronger via compensation. Furthermore, NMJs within muscle groups display striking differential vulnerability to denervation. For example, the levitor auris longus (a small muscle that controls ear movement in mice) displays diametric vulnerability. This muscle comprises two neighboring (rostral and caudal) fiber bands that are innervated by a single nerve bundle. While the rostral band is resistant to denervation, the caudal band readily degenerates (Murray et al., 2008), which suggests that intrinsic differences within motoneuron populations may determine susceptibility. Studies investigating motoneuron/NMJ vulnerability in SMA mouse models reported no correlation between susceptibility to denervation and muscle location, fiber type, nerve bundle length, NMJ size, axonal branching patterns, pruning rates, or Schwann cell expression (Ling et al., 2012; Thomson et al., 2012). While the mechanisms of vulnerability or resistance to denervation remain poorly understood, considerable effort is

ongoing to understand the regulating properties that determine susceptibility.

When do low levels of SMN cause neuromuscular dysfunction? Several thorough investigations of early embryonic development using SMA mice suggest that the neuromuscular system, even in the most vulnerable muscles, undergoes relatively normal establishment (Ling et al., 2012; McGovern et al., 2008; Murray et al., 2008), indicating that deterioration of NMJs after birth occurs in the absence of major developmental disturbances during neuromuscular establishment (Murray et al., 2008). This evidence suggests that denervation is a consequence of a failure to maintain the synapse, and that defects arise during neuromuscular maturation (Kariya et al., 2008). Restoration of SMN during early postnatal maturation in SMA mice (prior to denervation but after neuromuscular deficits are apparent) is able to reverse neuromuscular pathology (Lutz et al., 2011). In humans, denervation may present prior to birth, given detectable changes in fetal movements in SMA type 0-1 patients. This evidence further supports the conclusion that restoration of SMN during NMJ development should be a therapeutic target.

Maturational delays are another hallmark characteristic of SMA in mouse models and human tissue. Postsynaptically, endplates remain small and simple, lacking perforations or significant radial growth, and the switching of acetylcholine receptor subunits is delayed (Harding et al., 2015; Kong et al., 2009). In comparison, presynapses have poor terminal arborization and, in mouse models, delays in axonal pruning extend late into neuromuscular development.

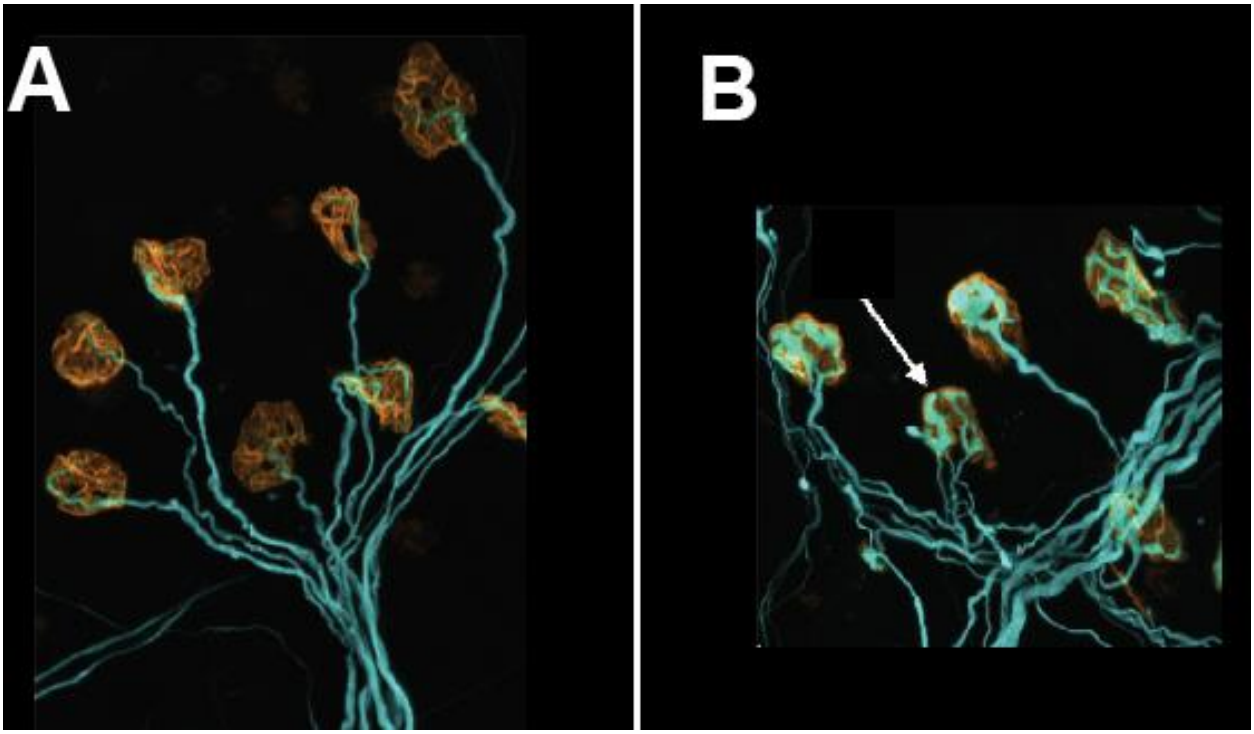

Figure 10 A comparison of neuromuscular development of the epitrochleoanconeus muscle between postnatal day 12 wildtype and SMA littermates.

(A) Endplates of wildtype mouse NMJs demonstrate intricately folded, perforated postsynaptic endplates that are innervated by single presynaptic axons. (B) SMNΔ7 mouse NMJs contain severe neurofilament blebs, tangled axons, poly-innervation (white arrow), and growth cones lacking endplates. Cyan, neurofilament, orange, α-bungarotoxin.

The most striking pathology, however, is the bulbous accumulations of neurofilament (predominantly in its phosphorylated form) (Kariya et al., 2008; Kong et al., 2009). Neurofilament accumulations in motor terminals appear to be a common feature of SMA and have been described in multiple mouse models. Interestingly, the presence of neurofilament blebs are observed in vulnerable and non-vulnerable muscles (Ling et al., 2012). These blebs are one of the earliest pathological alterations observed and increase with disease progression (Cifuentes-Diaz et al., 2002; Kariya et al., 2008; Murray et al., 2008), but the consequential pathogenic properties are

poorly understood.

Neurofilament accumulations have been observed in other neurodegenerative motor diseases, such as ALS and Charcot-Marie-Tooth, but this pathology is contained to somata or proximal axons (Dale & Garcia, 2012). This temporally and spatially regulated protein is synthesized in the soma and anterogradely transported to the terminal, undergoing various post-translational modifications, with phosphorylation being the most abundant form. Phosphorylation is critical for neurofilament organization and trafficking (Jung et al., 2005; Zheng et al., 2003), and de-phosphorylation permits disassembly and increases the affinity of neurofilament for dynein, a microtubule-based motor for retrograde transport (Perrot et al., 2008). Within terminals, dephosphorylated neurofilament is locally degraded by calcium-activated proteases prior to transport into the axon (Hisanaga et al., 1990; Sihag et al., 1999). SMNΔ7 mice show a decrease in dynein levels parallel to the time of neurofilament accumulation in terminals. These results suggest that axonal trafficking or transport impairment may be a primary disease mechanism in SMA and neurofilament pathology is a consequence of impaired local alterations in neurofilament dynamics (Dale et al., 2011). Disrupted β-actin mRNA transport, as seen in SMA mice, may also affect neurofilament shuttling and contribute to terminal blebbing (Bowerman et al., 2009; Bowerman, Shafey, & Kothary, 2007). Neurofilament blebs can consume a majority of terminal space, and thus may have secondary consequences including structural distortion of the terminal, displacement of organelles like mitochondria and vesicles, and disruption of neurotransmission.

Vulnerability to denervation and neurofilament blebbing are not the only pathologic features of SMA. Fewer active zones (Neve et al., 2016; Torres-Benito et al., 2011), synaptic vesicles (Dale et al., 2011; Kong et al., 2009; Neve et al., 2016; Torres-Benito et al., 2011), and vesicle release sensors (Dale et al., 2011) (Tejero et al., 2016) have been observed in vulnerable

and resistant muscles of mouse models. Reduced numbers of active zones have also been identified in post-mortem fetal tissue obtained from electively terminated pregnancies (Martínez-Hernández et al., 2013). Anterograde transport of SV2c and Syt1 (synaptic vesicle-associated proteins) is reduced prior to the decrease in vesicle populations in nerve terminals, despite expression levels in cell bodies remaining consistent (Dale et al., 2011). In addition, terminals have fewer mitochondria (Kong et al., 2009; Torres-Benito et al., 2011; Voigt et al., 2010), which is conjectured to be due to defects in transport (Miller et al., 2016; Xu et al., 2015) and consequently affects microtubule maturation (Torres-Benito et al., 2011). The reduced transport of key synaptic proteins may contribute to the breakdown and dysfunction of NMJs.

In addition to anatomical pathology, affected NMJs of severe mouse models display profound and persistent electrophysiological defects that appear during early stages of the disease, and are even in muscles resistant to denervation (Kong et al., 2009; Ling et al., 2010; Ruiz et al., 2010; Tejero et al., 2016). In highly vulnerable muscles of SMNΔ7 mice, such as the transverse abdominis or tibialis anterior, transmitter release is reduced by about 50% during the late stages of disease progression (Kong et al., 2009; Ruiz et al., 2010). Severe reductions in transmission are unlikely to satisfy the action potential threshold, and thus be insufficient to elicit a muscle fiber contraction. Additionally, impairments in endocytosis, a reduction in the probability of release, altered synaptic plasticity and smaller readily releasable and recycling synaptic vesicle pools (Kong et al., 2009; Tejero et al., 2016) have been reported in SMNΔ7 mice. Reductions in transmitter release, altered synaptic plasticity, and defective neuromuscular morphology were also seen in A2G mice, a milder mouse model of SMA (Ruiz & Tabares, 2014). Therefore, defective vesicle release appears to be a prominent consequence of SMN deficiency. Multiple mechanisms likely contribute to the reduction in transmission, but one intriguing hypothesis is a deficiency in

terminal calcium concentrations us responsible for the reductions in transmission.

Calcium homeostasis is an essential regulator of synaptic activity, and disruption in calcium dynamics has the potential to disturb NMJ integrity and induce pathophysiology. The first experimental support for the hypothesis of disrupted calcium homeostasis in SMA nerve terminals demonstrated aberrant clustering of VGCCs and decreased frequency of calcium transients in distal axons and growth cones, with no alterations apparent in cell bodies (Catterall, 2011; Jablonka et al., 2007). Application of cAMP, a second messenger that can increase the probability of calcium channel activation, increased the frequency of calcium transients and restored axonal function. Since these experiments, more evidence has surfaced supporting the hypothesis that altered calcium dynamics underlie neuromuscular pathophysiology. Reduced frequency of calcium transients in presynaptic terminals has also been observed in zebrafish models (See et al., 2014). *In utero* administration of *(R)*-roscovitine, a cdk5 inhibitor with VGCC agonist effects, increases the frequency of calcium transients and can recruit silent neuromuscular synapses in SMA mice via its mechanism of action on calcium channels (Tejero et al., 2020). Disrupted VGCC clustering has also been observed in NMJs of SMA mice (Ruiz et al., 2010). One potential explanation for impaired clustering of VGCCs is aberrant splicing (Doktor et al., 2017). Additionally, severely reduced expression of α-neurexins caused by SMN deficiency (as observed in SMA zebrafish and cultured motoneurons) may contribute to disrupted calcium homeostasis (See et al., 2014). α-neurexins selectively regulate calcium influx through Cav2.1 and Cav2.2 channels, which are the VGCCs associated with synaptic transmission at developing NMJs (Katz et al., 1996; Urbano, Pagani, & Uchitel, 2008; Urbano et al., 2003). Knock-outs of α-neurexins in wildtype mice results in electrophysiological defects similar to those observed in SMA mice (Missler et al., 2003; Zhang et al., 2005). In addition to reduced VGCC expression, aberrant splicing of CACNA genes, which

are responsible for the encoding of the pore-forming subunit in VGCCs, has also been reported (Doktor et al., 2017).

Several electrophysiological studies further support the hypothesis of aberrant calcium homeostasis in SMA motor terminals. NMJs of SMNΔ7 mice display a striking increase in calcium-dependent asynchronous release in response to prolonged stimulation (Ruiz et al., 2010). Furthermore, a comparison of synaptic transmission between control and SMA mice revealed no difference in transmission in low calcium conditions (1 mM), but significant impairment in SMA mice at physiological calcium levels (2 mM) (Tejero et al., 2016). Interestingly, application of PdBu, a DAG mimetic that drastically increases vesicle release from nerve terminals, increased quantal content in SMNΔ7 mice. This result suggests that, despite neurofilament aggregates and reduced vesicle populations, nerve terminals are functionally able to increase transmission. Furthermore, this evidence suggests that calcium homeostasis is a viable therapeutic target to improve neuromuscular function.

Curiously, several calcium-dependent protective modifiers of the SMA phenotype have been found through genomic transcriptome-wide differential expression analysis (Oprea et al., 2008). These SMN-independent modifiers bind, sense or regulate calcium and can improve neuromuscular pathology (Ackermann et al., 2013). Plastin 3 is an actin bundling protein that can positively modulates axonal outgrowth defects in zebrafish models, but only if the calcium-binding domain is intact (Lyon et al., 2014). Overexpression of Plastin 3 in motoneurons of SMA mice rescues synaptic vesicle populations in terminals and active zone defects (Ackermann et al., 2013), as well as increases neuromuscular transmission (Ackermann et al., 2013). Downregulation of CHP1, a protein that interacts with Plastin 3 and regulates the activity of the phosphatase calcineurin, improves axonal growth in NCS34 cells, SMA zebrafish, and SMA mouse motoneuron

cultures. In mouse models, CHP1 knockdown restores endocytosis (Janzen et al., 2018). Similarly, suppression of another modifier, Neurocalcin Delta, improves impaired endocytosis in SMA mice (Riessland et al., 2017).

Despite the extensive motoneuron-autonomous impairments caused by SMN deficiency, studies using muscle tissue from patients have demonstrated that SMN plays a significant role in muscular development. Significant abnormalities in myoblast and myotube differentiation have been observed (Guettier-Sigrist et al., 2001; Guettier-Sigrist et al., 2002), as well as delays in fiber growth (Martínez-Hernández et al., 2014), and AChR clustering (Arnold et al., 2004). Specific depletion of SMN in muscles of a mouse model causes morphological alterations to myofibers and NMJs, impaired motor function, and a shorter lifespan (Kim et al., 2020). Autopsy muscle tissue from patients (primarily at end stages of disease) shows atrophied fibers that are small, round and uniform with interspersed groups of relatively spared hypertrophic fibers. Hypertrophic fibers are usually type 1 (but see (Kingma et al., 1991)) and suggest a compensatory phenomenon. While myofiber atrophy is thought to be driven primarily by presynaptic withdrawal (Murray et al., 2008), it is clear that muscle-specific aberrations may contribute to NMJ pathology.

Unmyelinating Schwann cells may also influence neuromuscular pathology. Perisynaptic Schwann cells are reduced in number in SMA mice in vulnerable and resistant muscles (Neve et al., 2016), fail to completely cover endplate sites (Lee et al., 2011), and express fewer key proteins required to generate the peripheral extracellular matrix (Hunter et al., 2016; Hunter et al., 2014). Furthermore, selective restoration of SMN in Schwann cells improves neuromuscular function (Hunter et al., 2016).

The etiology of NMJ vulnerability is debated (Talbot & Davies, 2007), but distinguishing the contribution of each cell is important for directing therapeutic targets (Hamilton &

Gillingwater, 2012). Several studies have evaluated the specific consequences of selective SMN depletion (Braun et al., 1995; Gogliotti et al., 2012; Henderson et al., 1987; Hua et al., 2011; Hunter et al., 2016; Martinez et al., 2012; McCann et al., 2007) using genetically modified animal models or motoneuron-muscle co-cultures. Experimental findings suggest that SMN deficiency in motoneurons critically disrupts neuromuscular integrity, but skeletal muscles and Schwann cells potentially contribute to NMJ pathology as well (Boyd & Gillingwater, 2017; Hua et al., 2015). It is possible that dysfunctional spinal cord circuitry also influences neuromuscular pathology. However, restoration of the U12 splicing in motoneurons (an SMN-dependent RNA spliceosome pathway that is highly disrupted in SMA mice) restored proprioceptive contacts and improved motor function but failed to affect neuromuscular pathology (Osman et al., 2020), suggesting that local deficits are responsible for at least some of the neuromuscular dysfunction. In further support of multiple loci of dysfunction, postnatal reduction of p53 in an inducible intermediate SMA model rescues neuromuscular denervation but does not halt the degeneration of motoneuron somata (Courtney et al., 2019). The distinct pathogenic mechanisms in spinal cord and NMJ cells requires further investigation. However, evidence suggests that the neuromuscular circuitry is a treatment target to stabilize and protect NMJs and maximize the lifespan and motor function of SMA patients.

1.6.3 Pathophysiology caused by SMN deficiency in other tissues

In humans, the pathophysiological features of SMA are mainly restricted to the degeneration of lower α-motoneurons and the associated features of chronic motor axonal and synaptic loss. In exceptional cases, which comprise patients with the most severe forms of SMA

(one *SMN2* copy), defects in fetal cardiac development has been reported (Rudnik-Schoneborn et al., 2008). The most common abnormality is septal and cardiac outflow tract defects, which may contribute to rare but reported distal necrosis (Araujo, Araujo, & Swoboda, 2009; Parra et al., 2010). Benign cardiac arrhythmias have been reported in patients with milder forms of SMA (Wijngaarde et al., 2017). It is possible that these rhythmic abnormalities are, at least in part, a consequence of motor inactivity, as physical exercise has been shown to attenuate cardiac rhythmic dysfunction in SMA type 2 mice (Biondi et al., 2012). A variable degree of thalamic dysfunction (Ito et al., 2004) and thalamic neuronal degeneration and gliosis has also been reported in severe SMA patients at the end stage of the disease (Harding et al., 2015). Additionally, other abnormalities have been reported, comprising abnormalities in autonomic, sensory, gastrointestinal, and endocrine systems (Bowerman et al., 2014; Hamilton & Gillingwater, 2012; Harding et al., 2015; Porensky et al., 2012; Rudnik-Schoneborn et al., 2003; Rudnik-Schöneborn et al., 2010; Shababi et al., 2013; Simone et al., 2016). A number of organ phenotypes have been observed in mice. Abnormalities in cardiac, lymphatic, kidney, liver, pancreas, spleen, vasculature, bone and connective tissues have been extensively observed in animal models (thoroughly reviewed in (Yeo & Darras, 2020)). These non-canonical pathologies are generally reported in patients with the most severe forms of SMA, suggesting that even low expression levels of SMN (achieved by 2-3 functional copies of *SMN2*) in these tissues is sufficient for vitality.

Use of SMN-based genetic therapies in humans may uncover the extent of pathology in non-motor systems, particularly in severe SMA patients who would otherwise experience a robust motor phenotype and gravely shortened lifespan that could obfuscate other organ impairments not readily apparent during the natural disease progression.

1.7 Neuromuscular junctions are critical therapeutic targets in the treatment of SMA

Despite our current superficial understanding of the mechanisms regulating NMJ breakdown, a large and reproducible body of evidence supports the notion that neuromuscular junctions undoubtedly contribute to the disease pathogenesis in SMA. NMJ pathology and impairment is detectible during embryogenesis in mouse models and severe SMA patients, and precedes somal degeneration and clinical symptoms. Neuromuscular denervation induces the atrophy of muscle fibers, resulting in gradual weakening of and, in severe cases, eventual paralysis of vulnerable muscles. Furthermore, the temporal regulation and requirement of high levels of SMN is uncompromisingly restricted to the time period of neuromuscular development or repair. One study in a mouse model has shown that upregulation of even low levels of SMN in motoneurons is sufficient to mitigate neuromuscular denervation and pathology, while another study demonstrated that 15% of normal SMN levels in SMA mice prevented phenotypic onset until postnatal day 10 and moderately improved survival, though neuromuscular pathology was still evident (Bowerman et al., 2012; Paez-Colasante et al., 2013). Motoneuron-specific restoration of normal levels of SMN in mouse models re-established the synaptic properties of quantal content, innervation, endplate size, and neurofilament reduction (Martinez et al., 2012). These results suggest that the motoneuron- autonomous neuromuscular phenotype is highly sensitive to changes in SMN levels (Paez-Colasante et al., 2013).

Targeting calcium homeostasis and/or dynamics in motoneuron growth cones and mouse model NMJs during embryogenesis dramatically improves neuromuscular pathology and function. Application of (*R*)-Roscovitine, a cdk-5 inhibitor with minor positive allosteric effects on Cav2.1-2.2, increased Cav2.2 channel clustering, spontaneous calcium transients, elongated axons, and increased neurotransmission in SMA motoneuron cultures. Axonal elongation was similar when

motoneuron cultures were exposed to a derivative of (*R*)-Roscovitine, GV-58, which is a molecule with potent Cav2.1-2.2 effects with limited cdk activity (Tarr, et al., 2013). Additionally, systemic administration of (*R*)-Roscovitine to pregnant dams significantly augmented the lifespan of SMA mice. These effects were independent of effects on cdks, as (*S*)-Roscovitine, which lacks Cav2 activity but retains cdk-5 inhibition, did not improve lifespan (Tejero et al., 2020). These data suggest that calcium homeostasis in motor nerve terminals is crucial for motoneuron differentiation, maturation and function, and that targeting calcium dynamics during embryogenesis can remarkably alter neuromuscular pathology.

1.7.1 When is the optimal temporal intervention window to stabilize and protect NMJs?

Currently, no treatment options for SMA are administered prior to birth, but it is clear that neuromuscular symptoms begin during gestation for severe cases (which is the most common form of SMA). Treatment intervention depends upon diagnosis, which is almost always after birth and often after symptom manifestation. Therefore, therapeutic strategies that are aimed at treating patients already experiencing clinical symptoms may be limited by the inability to ameliorate or reverse established NMJ pathology. Is there a period during the development of neuromuscular pathogenesis that permits the reversal of pathology? To answer this question, mouse models have been used to determine the optimal time for therapeutic intervention.

Several sophisticated and detailed investigations have found that restoration of SMN using motoneuron-specific adeno-associated virus (AAV9) in SMA mice between postnatal day 0-2 effectively prevented the onset of disease and rescued most neuromuscular pathology. In comparison, AAV9 intervention on postnatal day 5 engendered modest benefits, while postnatal day 10 intervention did not deliver any benefit (Dominguez et al., 2011; Foust et al., 2010; Robbins

et al. 2014; Valori et al., 2010). One reason for the dramatic loss of benefits is due to the limited ability of the AAV9 to penetrate the blood-brain barrier and target motoneurons. To circumvent this problem, inducible SMN alleles in SMA mice have been utilized to elaborate our understanding of the temporal requirements of SMN-based therapy. SMN upregulation on postnatal day 1 greatly improved weight gain, survival and NMJ defects compared to SMN upregulation on postnatal day 2 (Le et al., 2011). In these mouse models, SMN levels peak around 3 days after intervention and suggest that the postnatal window of opportunity for maximal therapeutic advantage was between postnatal days 0-4 in severe model mice (Harding et al., 2015). One study using SMNΔ7 mice observed that small molecule-mediated correction of *SMN2* splicing from P3-23 prevented neuromuscular pathogenesis as far out as postnatal day 65 (Naryshkin et al., 2014). Similarities in the outcomes of the various studies investigating temporal requirements suggest that early intervention is sufficient to preserve neuromuscular integrity. These critical preclinical studies have dramatically influenced the structure of clinical trials for SMA. Additionally, clinical trials of SMN-based therapies support the conclusion that earlier intervention is better -- patients receiving treatment prior to symptom manifestation correlate with better disease outcomes.

1.7.2 The therapeutic quest to cure SMA

The last decade of preclinical research searching for curative treatments for SMA has developed significant advances in our understanding of the biologic, cellular, and genetic mechanisms of SMA. Despite this knowledge, several challenges have made drug development difficult. SMA comprises a broad spectrum of phenotypes, with a large population developing an onset of symptoms during infancy. Additionally, therapeutic treatment must be able to effectively

target disease-relevant tissue (such as lower α-motoneurons somas and NMJs). Preclinical investigations have shown that the best therapeutic approach to prevent disease progression is by increasing functional SMN levels. Slight differences of SMN expression in patients can substantially affect the phenotype, though this is not true for all patients (Prior et al., 2009). Additionally, animal models suggest that restoration of SMN later in life may still provide some therapeutic benefit (Le et al., 2011; Kariya et al., 2014; Lutz et al., 2011). Fortunately, studies suggest that even a relatively modest increase of SMN, when given early enough, would produce clinically meaningful improvements (Bowerman et al., 2012; Naryshkin et al., 2014; Paez-Colasante et al., 2013). Despite the temporal and cell-specific challenges, the first FDA approved, SMN-based treatment became available in December 2016, and in the four years since this approval, several promising candidates and one approved therapy has followed.

1.7.3 SMN-based therapies for SMA

Several SMN-based therapeutic approaches have been investigated to upregulate functional SMN protein. Examples of therapeutic approaches include targeting *Smn2* splicing (antisense oligonucleotides and other small molecules), transcription (histone deacetylase inhibitors, hydroxyurea, lncRNA-targeting oligonucleotides, prolactin, and quinazoline), translation (Indoprofen, aminoglycosides), as well as stabilization of SMN transcript or protein (Celecoxib) and the insertion of *SMN1* genes (adeno-associated viral and lentiviral vectors). Though only two SMA-modifying therapies have been FDA-approved (detailed in the following sections), SMN-based approaches have similarly altered the disease progression and outcome of patients with SMA. These types of therapies are currently the best method to prevent motoneuron degeneration (if administered early enough), but are not entirely curative for all patients receiving

treatment. In particular, patients with 1-2 copies of *SMN2*, and/or those who receive postsymptomatic intervention will likely require additional treatment strategies to improve residual dysfunction.

1.7.3.1 Nusinersen: an antisense oligonucleotide (ASO)

The endogenous presence of a paralog gene that produces the necessary protein has made *SMN2* an attractive therapeutic target. If splicing of *SMN2* is corrected to produce a greater percentage of full-length SMN, symptoms of SMA can be alleviated. The first FDA approved therapy for SMA uses small molecule (ASOs under the generic name nusinersen (brand name Spinraza)). Nusinersen uses a synthetic strand of nucleic acids linked together with a 2'O-methoxyethyl backbone that functions by recognizing and binding to cellular RNA to correct gene splicing. Nusinersen use Watson-Crick pairing to specifically bind the ISS-N1 (intronic splicing silencer-N1) sequence in *SMN2*, which is a major inhibitory element regulating the splicing of exon 7. ISS-N1 has proven to be a model target for ASOs to increase the ratio of full-length SMN protein derived from *SMN2* transcripts because ISS-N1 inhibition results in the inclusion of exon 7 in *SMN2* transcripts (Singh et al. 2017).

Upon intrathecal administration, ASOs are endocytosed by cells, though the mechanisms regulating this are poorly understood. Upon entrance to the cell body, nusinersen ASOs enter the nucleus where they bind to *SMN2* pre-mRNA transcripts. Advancements in technology, such as more penetrative and durable ASO chemistries and better therapeutic safety and efficacy, have drastically altered the outcome and progression of this previously unmodifiable neuromuscular disease (Verma, 2018).

Preclinical studies in mice have shown that administration of ASOs targeting ISS-N1 is sufficient to mitigate neuromuscular pathology and greatly improve survival and motor function

in a time- and dose-dependent manner (Hua et al., 2010; Hua et al., 2011; Passini et al., 2011; Passini et al., 2010; Porensky et al., 2012; Zhou et al., 2013). In nonhuman primates, a single intrathecal injection of nusinersen sufficiently increased tissue concentrations of full-length SMN for several months (Rigo et al., 2012), suggesting that the long half-life of the drug permits several months between treatments for SMA patients (d'Ydewalle & Sumner, 2015). Indeed, patient autopsy results demonstrate that intrathecal delivery of nusinersen elevates *SMN2* mRNA exon 7 inclusion in neurons and other cell types in the spinal cord and brain. Clinical studies of nusinersen in all types of SMA patients showed encouraging clinical efficacy, tolerability, and pharmacology consistent with its intended mechanism of action. Intrathecal injections were well tolerated and improved motor function in a majority of treated patients (d'Ydewalle & Sumner, 2015; Finkel, Mercuri, et al., 2017; E. Mercuri et al., 2018). Type 1 patients had remarkably improved lifespans compared to what would have been normally expected from the natural history of the disease, and experienced fewer respiratory complications requiring ventilation (Finkel et al., 2017). More historical data is necessary to determine the effects on the lifespan of individuals with less severe forms of SMA. ASO treated patients achieved motor milestones that would have been unprecedented in the natural disease progression, and many achieved these skills within normal time frames of motor development.

Patients with later-onset SMA that have received ASO therapy report a better quality of life. Caregivers of these patients also report a decreased impact of caring burden and also report a greater quality of life (Sanchez et al., 2019). Despite these benefits, a high percentage of both ambulatory and non-ambulatory adult patients receiving nusinersen continue to show decreased compound muscle action potentials after 14 months of therapy, indicating that neuromuscular function remains compromised (WArnold, 2020). The decrease in neuromuscular transmission

correlates with reduced motor performance in a fatigue test, suggesting that NMJ function is as promising target for additive pharmacological interventions.

Intrathecal administration is relatively invasive, requiring sedation or anesthesia, and can be particularly problematic in young patients or patients with scoliosis. Since ASOs have a limited penetrability of the blood-brain barrier, they require direct administration into the cerebrospinal fluid to achieve endocytosis into motoneuron somata. Penetrance into peripheral systems is restricted, however (Bowerman et al., 2017). Primarily central administration is problematic because mouse studies have established that peripheral SMN upregulation is essential for the long-term improvement of peripheral pathologies (Hua et al., 2011; Lee et al., 2012; Paez-Colasante et al., 2013). However, systemic delivery of ASOs is associated with a higher risk of toxicity due to a requirement of a much larger dose (Banks et al., 2001). In addition, the fragile nature of patients' pulmonary and respiratory status requires predetermined strategies to optimize systemic function and contingency plans for potential complications, thus potentially delaying treatment (Neil & Bisaccia, 2019). Delays may result in treatment onset after the optimal therapeutic window, particularly for patients who are diagnosed after symptom onset.

In the United States, nusinersen is currently approved for the treatment of any patient with biallelic mutations or deletions of *SMN1* (Corey, 2017). During the first year of treatment, nusinursen is estimated to cost $750,000 for the six prescribed doses. Thereafter, the medication costs $375,000 for the recommended three maintenance doses per year. No cessation of annual treatments is recommended *per se*, unless the patients (or their caregivers) elect not to continue treatment. The medication costs do not include the additional and substantial medical costs associated with treatment (including but not restricted to medical appointments and follow-ups, administration costs, and invisible costs incurred by caregivers). Outside the United States,

widespread approval for use of ASOs as a therapy for SMA has been slow, primarily hampered by the high price tag of the drug (Neil & Bisaccia, 2019).

ASOs have remarkably altered the predicted disease outcome for SMA patients. These molecules are relatively simple to manufacture, have little toxicity when administered centrally, and demonstrate a high target specificity, resulting in better motor function and, in some cases, significantly longer lifespans. However, the therapeutic enhancement of SMN induced ASOs is not sufficient to mitigate all pathology (Finkel et al., 2017; Mercuri et al., 2018). ASOs may fail to provide sufficient elevation of SMN required by motoneurons in patients with only 1-2 copies of *SMN2*, resulting in a vulnerability to age or injury related deterioration over the patient's lifetime. These limitations would be magnified for patients who receive delayed or postsymptomatic therapeutic interventions. Lastly, financial (such as insurance premiums and/or deductibles, or switching of insurance policies), access (specialist referral requirements, insurance-dictated eligibility, or physical ability to reach certified outpatient facilities), or emotional (such as anxiety or depression associated with medical visits) constraints may prevent a patient from receiving all recommended doses, therefore restricting the long-term benefits induced by ASOs. While many U.S. insurance companies have decided to cover nusinersen therapy, some companies have implemented stricter guidelines for patient eligibility than recommended by the FDA, such as age limitations, requirement of symptom manifestation prior to treatment coverage, or restricting access for patients with greater than 2 *SMN2* copies (Pagliarulo, 2019). These company-imposed restrictions are due to the high cost of nusinersen. A report released by the Institute for Clinical and Economic Review comparing the quality-adjusted life-year (an economic evaluation used to assess the value of a medical intervention (such as the cost of nusinersen) in disease burden, assessing both quality and quantity of life) suggests that the nusinersen list price should be

discounted 10-fold (Ellis et al., 2019; Starner & Gleason, 2019). This dissonance in price and benefit is important to consider because substantial medical costs (whether due to out-of-pocket medical costs and/or income loss due to illness or time off work in the case of caregivers) significantly correlates with filing for bankruptcy in the United States (Dobkin et al., 2018; Himmelstein et al., 2009; Himmelstein et al., 2005).

1.7.3.2 Onasemnogene Abeparvovec-Xioi: a self-complimentary adeno-associated virus (scAAV9)

The second approved therapy for SMA utilizes recombinant adeno-associated virus vectors to target the central underlying defect caused by the disease. Available to patients under 2 years of age with biallelic mutations or deletions of *SMN1*, onasemnogene abeparvovec-xioi (brand name Zolgensma) is a genetic therapy that utilizes a fully functional copy of human *SMN1* packaged into an AAV9 capsid. Targeting of cells within the central nervous system is achieved by the use of the AAV9 serotype, which has been shown to efficiently transduce within neurons and glia in rodents and non-human primates. This medication only requires a single intravenous administration over the period of 60 minutes to achieve therapeutic benefit, as this scAAV9 has robust transduction efficiency and a sustainable presence in host cells. Upon intravenous delivery, the non-replicating scAAV9 crosses the blood-brain barrier into the central nervous system, where it is endocytosed by motoneurons and trafficked to the nucleus. Once in the nucleus, the virus uncoats and transduces the host cell to transcribe its double-stranded DNA unit (*SMN1*) (McCarty, Monahan, & Samulski, 2001). Zolgensma is designed not to integrate into the host genome; therefore, patients receiving this therapy would still test negative for the presence of functional *SMN1* (Bevan et al., 2011; Cearley & Wolfe, 2006; Dayton, Wang, & Klein, 2012; Foust et al., 2009; Gray et al., 2011).

Studies in neonatal mice have demonstrated that peripheral administration of GFP-tagged scAAV9 with a chicken β-actin promoter was able to transduce in 60% of motoneurons, and was measurable 20 days after injection (Foust et al., 2009). However, administration to postnatal day 10 mice had limited effects, further supporting the need for early intervention. When tested in newborn cynomolgus macaques, peripherally administered scAAV9 crossed the blood-brain barrier and transduced in motoneurons (Bevan et al., 2011; Gray et al., 2011). However, unlike mice, intravenous administration of GFP-tagged scAAV9 administration to monkeys as old as 3 years was still able to transduce in CNS cells, though mostly microglia and astrocytes (Bevan et al., 2011). Furthermore, the scAAV9 was detectible in peripheral muscle tissue of all age groups, though older monkeys had weaker expression. These studies suggest that intravenous scAAV9 administration to adults with SMA may have restricted expression in motoneurons. Ongoing studies are exploring methods to improve viral penetrance (Meyer et al., 2014).

When administered to SMNΔ7 mice, scAAV9 using a chicken β-actin promoter can elevate SMN levels in disease-relevant tissues, improve motor function, and dramatically improve lifespan (Foust et al., 2010). However, scAAV9 with a synapsin promoter to narrowly target neurons and exclude peripheral organs did not rescue neuromuscular pathologies or survival in SMA mice (Besse et al., 2020), suggesting that peripheral defects contribute to the disease severity, and thus ubiquitous SMN expression is necessary to achieve full symptomatic improvement (Hamilton & Gillingwater, 2012; Shababi et al., 2013). Zolgensma uses a chicken β-actin hybrid promoter with a cytomegalovirus enhancer to improve SMN upregulation in motoneurons (Gray et al., 2011).

Clinical trials of Zolgensma have demonstrated remarkable improvement of motor function in SMA patients. For patients with 3 copies of *SMN2*, patients achieved motor skills within normal age ranges of acquisition. In comparison, a majority (but not all) of patients with 2 copies of *SMN2*

also achieved motor milestones within normal ranges. A long-term follow-up of patients who were treated postsymptomatically showed that no patients lost any achieved motor skills, which is a stark contrast to the natural disease history. The most common achieved motor milestone included head control, rolling from back to side, and sitting unsupported for more than 30 seconds. However, less than half of the patients receiving a postsymptomatic intervention achieved advanced motor skills such as walking or standing (supported or unsupported) (Lowes et al., 2019). Additionally, Zolgensma dramatically improved respiratory function regardless of pre or post-symptomatic administration. In comparison, over 90% of patients with severe forms of SMA not receiving SMN-based therapy require permanent ventilation by their second birthday.

While gene therapy has remarkably changed the field of SMA treatment, use of Zolgensma is not without potential drawbacks. The inherited prevalence of maternally-derived (potentially transient) neutralizing antibodies to AAV9 may restrict the use of this type of therapy due to the risk of rendering the viral vector ineffective (Halbert et al., 2006). Furthermore, gene transfer does not permit dose cessation if a safety issue arises. In particular, Zolgensma has a potential risk of liver and cardiac complications, and requires monitoring of liver and cardiac function prior to and for 3 months after infusion ("ZOLGENSMA [package insert]," 2019). However, the scAAV9 is a non-replicating virus, so regenerative tissues like the liver are unlikely to retain prolonged SMN upregulation. Risk for immune response or peripheral dysfunction would also delay or prevent gene therapy. Additionally, unlike antisenese oligonucleotides with a mechanism of action on the endogenous *SMN2* gene(s), AAV9 vectors do not have an inherent ceiling of SMN upregulation. Possible long-term consequences of continuous AAV9-induced SMN expression are currently being investigated. One study utilizing a subthreshold AAV9 dose in wildtype SMA mice induced the deterioration of proprioceptive contacts in the spinal cord, resulting in a hind limb-clasping

phenotype in postnatal day 300 mice. This hind limb clasping phenotype was also observed in AAV9-treated SMA mice, and suggests that perpetual SMN upregulation could induce late-onset impairments of sensory-motor circuits (Van Alstyne, 2020). A final consideration should also be given to the potentially prohibitive cost of Zolgensma -- at $2.1 million for a single dose, this medication is the world's most expensive drug (Dyer, 2020). Similar to insurance company-imposed restrictions mentioned in 1.7.3.1, limitations of age, symptom onset, and/or *SMN2* copy numbers caused by the high price of Zolgensma restrict many patient's eligibility for this therapy through their insurance company, despite the suggestion from the Institute for Clinical and Economic Review comparing the quality-adjusted life-year to discount the Zolgensma list price by 2-fold (Ellis et al., 2019; Starner & Gleason, 2019).

1.7.3.3 The advent of new SMN-dependent treatments

Despite the remarkable benefits of these approved therapies, invasive central administration (in the case of ASOs) or restricted patient eligibility and inability to cease use (in the case of AAVs) have provided a window of opportunity for new SMN-based therapies. One of these therapies utilizes an orally administered small molecule, RG7916 (Risdiplam), to correct *SMN2* splicing. Risdiplam is currently in Phase 1/2 clinical trials and has been shown to increase SMN protein in whole blood (Kletzl et al., 2018). While benefits to motor function and lifespan of patients is still under investigation, oral use and peripheral dissemination make this SMN-based therapy an attractive competitor. Similar to other SMN-based medicines, however, Risdiplam is unlikely to provide full symptomatic relief for patients with delayed interventions or low copy numbers of *SMN2*.

1.7.4 SMN-independent therapies

While SMN-based therapies remain the forefront of promising therapeutic intervention, evidence suggests that additional treatments will be necessary to address persistent deficits. An extensive number of therapeutic strategies have been investigated, targeting a wide range of affected cells to protect and improve function. Furthermore, considering the narrow temporal requirements of SMN, SMN upregulation in adolescent or adult patients may have limited benefits. Therefore, therapeutic strategies that can be widely used across patient populations of variable ages are crucial.

1.7.4.1 Neuroprotective strategies

Neuroprotective strategies encompass SMN-independent treatments that are targeted to prevent dysfunction in motoneurons and associated circuitry. While several neuroprotective treatments have been previously attempted in other neurodegenerative diseases, such as Alzheimer's, Parkinson's disease, and amyotrophic lateral sclerosis, relatively poor outcomes of clinical trials have limited widespread investigations of neuroprotective agents for SMA. Neurodegeneration is a complex, multicellular process and consequently targeting single cell pathways contributing to death or survival may not be sufficient to halt or improve disease progression.

There are two neuroprotective agents that have been approved for use in other diseases have been tested for efficacy in SMA patients. Gabapentin, an anticonvulsant used to treat neuropathic pain and restless leg syndrome, has been evaluated due to its ability to decrease glutamate signaling and thus decrease excitotoxicity. However, two large clinical trials composed of type 2-3 SMA patients demonstrated minimal effects on motor function (Merlini et al., 2003;

Miller et al., 2001; Wadman et al., 2020). Another drug, riluzole, has been used to treat ALS patients with some benefits. ALS patients receiving this drug have lifespans extended by 2-3 months, though with no benefit to motoneuron function. Use of riluzole in SMA animal models appeared promising, where it was able to improve neuromuscular defects in c. elegans model (Dimitriadi et al., 2013), and lifespan and cytoskeletal organization in mouse models (Haddad et al., 2003). However, a small clinical trial comprising 10 SMA type 1 infants (7 riluzole-treated, 3 placebo-treated) was cut short due to lack of improvement in motor milestones, though the small cohort underpowers statistical assessment. A second clinical trial (clinicaltrials.gov: NCT00774423) evaluated the effects of 50 mg of riluzole in type 2-3 patients, which has been shown to be a sufficient dose for pharmacokinetic exposure in younger SMA patients (Abbara et al., 2011). However, results of this trial have yet to be published (as of June 2020).

One neuroprotective approach that has been evaluated in SMA models utilizes enhancers or mimetics of neurotrophic factors. Around 15 endogenous trophic factors have been discovered that modulate motoneuron survival *in vitro*, including but not limited to brain-derived neurotrophic factor (BDNF), neurotrophin-3, vascular endothelial growth factor, glial cell-derived growth factor (GDNF), ciliary neurotrophic factor (CDNF), and insulin-like growth factor-1 (IGF-1). Historically, BDNF, GDNF, and CDNF have been extensively investigated to prevent motoneuron degeneration and improve motor function (Guo et al., 2019; Haase et al., 1997; Mitsumoto et al., 1994; Oppenheim et al., 1995; Sendtner et al., 1992; Sendtner et al., 1992; Sendtner, Stockli, & Thoenen, 1992). However, much of the work in the SMA field has focused on IGF-1 and BDNF.

Low levels of IGF-1 have been detected in severe SMA mouse models, which are restored to normal levels upon correction of SMN levels (Hua et al., 2011; Murdocca et al., 2012). Overexpression or mimetic administration of IGF-1 to SMNΔ7 mice improves lumbar motoneuron

degeneration, cardiac defects, fiber size, and motor function, but has limited benefits on survival (Bosch-Marcé et al., 2011; Murdocca et al., 2012; Shababi, Glascock, & Lorson, 2011; Tsai et al., 2014). Administration of AAV-driven IGF-1 into deep cerebellar nuclei of intermediate SMA mice improves motoneuron degeneration but has no effects on neuromuscular pathology (Tsai et al., 2012). These studies indicate that IGF-1 may be a potential target for neuroprotective approaches, but are likely to have restricted benefits if used without complementary treatments.

The effects of BDNF have also been explored in developing motoneuron cultures and SMA models. Through its mechanism of action on TrkB receptors, BDNF application increases calcium transients via increased Cav2.2 clustering, and increases F-actin assembly and growth cone formation in motoneurons *in vitro* (Dombert et al., 2017), suggesting that some of the developmental defects seen in SMA model cultured motoneurons (Jablonka et al., 2007) and mice (Tejero et al., 2020) are consequences of reduced BDNF-mediated trophic support. SMN-regulated BDNF expression has also been explored in SMA model NSC34 motoneuron-like cells. SMN deficiency results in the downregulation of Akt pathway, which is regulated downstream by BDNF binding to TrkB (Numakawa et al., 2010). Application of loganin, a neuroprotective iridoid glycoside, to NSC34 cells increased neurite length, cell viability, and SMN expression, upregulates BDNF and activates the Akt pathway in these cells. Loganin administration to SMNΔ7 mice improved motor function and mildly improved lifespan, and blockade of the IGF-1 receptor attenuated the protective effects of loganin (Tseng et al., 2016). Agrin is another trophic factor that is significantly reduced in SMA mice. Administration of NT-1654, a cleavage-resistant splice variant of z^+ agrin with synaptogenic properties, significantly improved motor function and neuromuscular pathology (Boido et al., 2018). The mechanism(s) regulating the reduction of trophic factors in SMN-deficient systems requires additional investigation, but nevertheless may

be therapeutic targets to improve the integrity of motor circuits.

1.7.4.2 Muscle-directed strategies

Given the intrinsic role of SMN in muscular development, strategies that enhance muscle function have been explored. One of the most investigated targets with promising results is the inhibition of myostatin. Myostatin is a member of the transforming growth factor B superfamily, and is predominantly synthesized and expressed in skeletal muscle. Myostatin acts as an endogenous negative regulator of skeletal muscle growth and size (Lee & McPherron, 2001; McPherron, Lawler, & Lee, 1997; Zhu et al., 2000), and is neutralized by the autocrine glycoprotein follistatin and myostatin propeptide (Hill et al., 2002). Studies evaluating the benefits of recombinant follistatin administration to SMNΔ7 mice observed improvements in lifespan, motor function, and motoneuron death (Rose et al., 2009). However, other studies inactivating follistatin in SMNΔ7 mice did not significantly ameliorate motor function, fiber size, or survival (Rindt et al., 2012; Sumner et al., 2009), likely due to the rapid disease progression in this mouse model. One study evaluating AAV-driven myostatin inhibition in SMNΔ7 mice treated with SMN-based ASOs observed improvements in weight gain, motor function and endurance, survival, proprioceptive synapses onto motoneurons, and mitigated neuromuscular pathology (Zhou et al., 2020). Another study evaluating myostatin inhibitors in mild SMA mice demonstrated efficacy in late disease stages (Feng et al., 2016). Myostatin is also a therapeutic target for SRK-015, which is a monoclonal antibody that blocks the activation of the latent form of myostatin rather than inhibiting the mature myostatin form or blocking its receptor (Dagbay et al., 2020). Preclinical studies in SMA mice has shown that both early and late administration of muSRK-015P (a suboptimal variant of SRK-015) increases muscle mass and motor function (Long et al., 2019). Three clinical trials are underway assessing the benefits of intravenously administered SRK-015,

primarily for use as a complementary SMN-based therapy. This treatment is one of the first muscle-directed therapies to improve muscle atrophy in a broad range of SMA types.

Another muscle-centric approach utilizes fast skeletal troponin activators. Troponin complexes regulate contraction in skeletal and cardiac muscles. Fast skeletal troponin activators increase calcium affinity of the troponin-tropomyocin regulatory complex, which results in the sensitization of the sarcomere to calcium concentrations and improves skeletal muscle contractility and muscle performance. Force-calcium and force-frequency relationships are leftward shifted by troponin activation. Thus, increased contractility is observed even with inadequate neural signaling because these activators amplify the response of the muscle to sub-maximal nerve stimulation (Russell et al., 2012). Clinical trials for tirasemtiv, a fast skeletal troponin activator, have completed phase 2 of clinical trials, but results have not been published (as of June 2020).

1.7.4.3 Drugs targeting neuromuscular transmission

Reduced neuromuscular transmission and increased NMJ failure is characteristic of the SMA phenotype and has led to the testing of drugs that improve neuromuscular communication. However, few drugs have provided symptomatic relief for SMA patients (Wadman, der Pol, & Vrancken, 2011). Pyridostigmine, an acetylcholinesterase inhibiter canonically prescribed to patients with myasthenia gravis, was reported to increased stamina in 2 of 4 SMA type 2-3 patients (der Pol et al., 2012). Though limited, this positive finding supported the evaluation of pyridostgmine in a clinical trial comprised of patients with SMA types 2-4 living in the Netherlands (Clinicaltrials.gov: NCT02941328). While the trial was completed in 2018, results have yet to be published (as of June, 2020).

Similarly, the voltage-gated potassium channel antagonist 3,4-DAP is also undergoing two clinical trials for patients with SMA type 3, though both trials are currently in the recruiting phase

(Clinicaltrials.gov: NCT03781479, NCT03819660). 3,4-DAP is canonically prescribed to patients with Lambert-Eaton myasthenic syndrome (LEMS), which is a neuromuscular disease similarly characterized by a reduction in presynaptic transmitter release. Unlike pyridostigmine, 3,4-DAP is poorly tolerated due to dose-dependent side effects, and low doses often do not produce full symptomatic relief for patients with LEMS. Another voltage-gated potassium blocking drug being tested in clinical trials is 4-aminopyridine. Similar to 3,4-DAP, 4-AP has dose-dependent side effects that restrict the use of large doses. 4-AP has traditionally been used to treat patients with multiple sclerosis due to its more efficacious penetrance of the blood-brain barrier compared to 3,4-DAP. Results from these clinical trials have also not yet been published (Wadman et al., 2020).

1.7.4.4 Endogenous SMN-independent protective modifiers

Transcriptome-wide differential expression analysis of genes from SMA-discordant families have elucidated endogenous protective modifiers of the SMA phenotype (Oprea et al., 2008). Curiously, several of these modifiers bind calcium and function by sensing or regulating calcium. These positive modifiers include plastin 3 (PLS3) (Ackermann et al., 2013; Hosseinibarkooie et al., 2016; Oprea et al., 2008), neurocalcin delta (NCALD) (Riessland et al., 2017), and calcineurin-like EF-hand protein 1 (CHP1) (Janzen et al., 2018).

Downregulated neurocalcin delta (NCALD) is a protective modifier of the SMA phenotype and functions as a neuronal calcium sensor to negatively regulate endocytosis. Heterozygous knockdown of NCALD improves endocytosis in fibroblasts derived from SMA patients. Knockdown also improves axon elongation and NMJ size in severe and mild SMA mice, and accelerates neuromuscular maturation and improves motor function in intermediate SMA mice. Additionally, suppression of NCALD improves proprioceptive contacts onto motoneuron somas (Riessland et al., 2017). A dual approach to treating SMA has been evaluated by combining SMN-

increasing ASOs and NCALD-reducing ASOs in severe SMA mice. Compound muscle action potentials, motor unit numbers, muscle fiber size, and grip strength were preserved when these two treatments were combined, compared to the use of SMN-ASOs alone (Torres-Benito et al., 2019).

PLS3 was the first identified positive modifier of the SMA phenotype (Oprea et al., 2008), and was found to be highly upregulated in differentiated motoneurons obtained from fibroblasts of discordant siblings (Oprea et al., 2008). High levels of PLS3 protect individuals with 3-4 copies of *SMN2* from SMA onset even in the presence of biallelic *SMN1* deletion. PLS3 is located on chromosome Xq23 and is a calcium-dependent F-actin-bundling protein that modulates the cytoskeleton, axonal growth and migration, vesicle trafficking, endocytosis, and regulates the F/G actin ratio (Alrafiah et al., 2018; Delanote, Vandekerckhove, & Gettemans, 2005; Engqvist-Goldstein & Drubin, 2003; Pollard & Borisy, 2003). Mutation of the calcium-binding residues within the EF hands of PLS3 abolishes the positive modifying effects of PLS3 in a zebrafish SMA model (Lyon et al., 2014). Overexpression of PLS3 in SMN-deficient motoneurons and SMA morpholino zebrafish restores axonal growth and motor function, (Oprea et al., 2008; Alrafiah et al., 2018; Hao et al., 2012), and improves the survival of mild and severe SMA mice (Alrafiah et al., 2018; Hosseinibarkooie et al., 2016; Kaifer et al., 2017). At the level of the spinal cord, PLS3 overexpression in SMA mice increased motoneuron soma size and the number of proprioceptive synapses (Ackermann et al., 2013). In NMJs, PLS3 upregulation in animal models corresponds with augmented neurotransmission (Ackermann et al., 2013), restored endplate and muscle fiber size, improved vesicle trafficking and nerve terminal accumulation, restored endocytosis and actin dynamics, and increased the number of terminal active zones (Ackermann, et al., 2013; Hao et al., 2012; Hosseinibarkooie et al., 2016). Additionally, PLS3 stabilizes synaptic innervation, resulting

in delayed axonal pruning in NMJs of SMA mice (Ackermann et al., 2013), thus improving the weakened nerve-muscle connection characteristic of SMA.

PLS3 has several binding partners, one of which is the calcineurin inhibitor CHP1. CHP1 dephosphorylates proteins involved with calcineurin phosphatase activity and has elevated expression in SMA mice. Knockdown of CHP1 restored axonal growth in SMN-depleted NCS34 motoneuron-like cells, SMA zebrafish, and primary SMA mouse motoneuron cultures (Janzen et al., 2018). In SMA mice treated with SMN-based ASOs, CHP1 reduction prolonged survival, improved electrophysiological defects and NMJ growth and maturation, and muscle fiber size in comparison to the effects of ASOs alone. In addition to CHP1, PLS3 also binds to CORO1C in a calcium-dependent manner to mediate endocytosis and actin dynamics (Hosseinibarkooie et al., 2016). This evidence suggests that actin dynamics in motoneurons is a highly calcium-dependent process that strongly modulates disease pathogenesis by increasing neuromuscular function and stabilizing motoneuron circuitry.

1.7.4.5 Physical therapy strategies

The benefits of exercise have been evaluated in SMA mice and patients. In intermediate SMA model mice, elevated levels of full-length SMN have been observed after either acute or chronic exercise (Grondard et al., 2008; Ng, Mikhail, & Ljubicic, 2019). Furthermore, chronic exercise significantly improved motoneuron maturation and somal loss in the lumbar spinal cord (Biondi et al., 2008) and extended lifespan (Grondard et al., 2005), suggesting that exercise can mitigate pathology and disease progression. Elevated levels of IGF-1 have been observed after exercise in SMA mice, potentially providing neuroprotective support through a trophic action rather than through simple muscle size improvement (Charbonnier, 2007). However, these trophic and SMN upregulation benefits were not observed in patients in arm cycling exercise training

programs for type 2 patients (Bora et al., 2017). Other studies evaluating the benefits of physical exercise in mild SMA mice found improvement in glucose homeostasis and oxygen consumption, and improvement in muscle mitochondria function (Houdebine et al., 2019), especially when the exercise was high-intensity. Chronic exercise in these mice improved muscle fatigue, neuromuscular excitability, and increased the resistance of muscles to damage (Chali et al., 2016).

Evaluation of exercise in patients has been primarily reported on type 2-3 patients not receiving SMN-based therapies. Rehabilitative interventions for SMA patients include physical therapy, strengthening and balance exercises, aquatic therapy and physical activity. Most published reports of the benefits of exercise in patients have been individual case studies (Lewelt et al., 2015), though some clinical trials have been initiated. For example, cycle ergometer training in type 3 patients has been demonstrated to improve oxidative capacity but induces significant fatigue (Madsen et al., 2015). Other programs have utilized at-home strength and aerobic exercise trainings to improve motor function, strength, fatigue and cardiovascular fitness in patients. Benefits of these exercises (in particular, aerobic exercise) have been difficult to assess due to a high drop-out rate (Bartels et al., 2019). However, exercise through sport activity has been shown to significantly improve self-esteem and identity, reduce depression, and result in a greater quality of life for patients with neuromuscular diseases, including spinal muscular atrophy patients (Vita et al., 2020). With new FDA-approved SMN-based therapies improving the ability of patients to participate in activity that demands endurance, strength, and motor skills, exercise may be an excellent, low-cost and accessible method to improve the negative social and emotional aspects of SMA.

2.0 3,4-Diaminopyridine broadens action potentials to increase transmitter release at the neuromuscular junction independent of Cav1 calcium channels

2.1 Introduction

Lambert-Eaton Myasthenic syndrome (LEMS) is a neuromuscular disorder caused by an autoantibody-mediated attack on the presynaptic Cav2.1 type (also called "P/Q-type") voltage-gated calcium channels (and other presynaptic proteins) at the neuromuscular junction (Lambert, Eaton, & Rooke, 1956; Lang, Molenaar, Newsom-Davis, & Vincent, 1984; S. D. Meriney, Hulsizer, Lennon, & Grinnell, 1996; Nagel, Engel, Lang, Newsom-Davis, & Fukuoka, 1988; Tarr, Malick, et al., 2013; Vincent, Lang, & Newsom-Davis, 1989). This results in reduced acetylcholine release from the NMJ, which leads to a failure of some postsynaptic muscle fibers to initiate an action potential (AP), resulting in a weaker muscle contraction. Clinical and animal model studies suggest that neuromuscular function and subsequent muscle strength can be improved by the use of 3,4-diaminopyridine (3,4-DAP), which is a small molecule that targets voltage-gated potassium channel function. 3,4-DAP was recently approved by the FDA to treat LEMS (FDA press release, 2019; FDA press release, 2018; Voelker, 2019; Yoon et al., 2020) and has been shown to be effective at increasing neuromuscular strength in LEMS patients (Oh, 2016; Sanders et al., 2018; Shieh et al., 2019; Strupp et al., 2017). Although 3,4-DAP does not penetrate the blood-brain barrier well, it still has dose-dependent side effects that prevent full symptomatic relief in many LEMS patients (Lindquist & Stangel, 2011; Wirtz et al., 2009). Patients are typically prescribed 10-20 mg oral doses of 3,4-DAP to be taken several times during the day, and report peak clinical effects after each dose for 3-8 hours (Sanders, 1998). Pharmacokinetic studies cite peak serum

concentrations of ~40-110 ng/ml after a 20 mg oral dose that has been reported to have a serum half-life of 1-3 hours (Haroldsen, et al., 2015; Haroldsenet al., 2015; Kobayashi et al., 2015; Thakkar et al., 2017; Wirtz et al., 2009). Similar doses of 3,4-DAP have also been used off-label for a variety of other neuromuscular weakness conditions, including congenital myasthenic syndrome (Banwell et al., 2004; Natera-de Benito et al., 2016a; Natera-de Benito, et al., 2016b; Rodríguez Cruz et al., 2016; Rodríguez Cruz et al., 2019; Witting et al., 2015), muscle-specific receptor tyrosine kinase (MuSK) (Bonanno et al., 2018), downbeat nystagmus (Thurtell & Leigh, 2012), and multiple sclerosis (Flet et al., 2010; Polman et al., 1994; Sheean et al., 1998; Mainero et al., 2004).

The mechanism of action of 3,4-DAP is canonically thought to be a block of Kv3 type (also called "A-type") voltage-gated potassium channels. Kv3.3 and Kv3.4 channels are the subtypes selectively localized in mammalian neuromuscular motor nerve terminals (Brooke et al., 2004) and are thought to be predominantly responsible for speeding the repolarization of the presynaptic AP. Blocking Kv3 channels and broadening the presynaptic AP increases the number of presynaptic Cav2 voltage-gated calcium channels that open within transmitter release sites, and this results in an increase in the calcium influx that triggers acetylcholine release. Because calcium-triggered acetylcholine release is non-linearly dependent on calcium concentration in nerve terminals, a relatively small increase in calcium ion entry into nerve terminal can generate a much larger increase in neurotransmitter release (Dodge & Rahamimoff, 1967).

More recent investigations have challenged the conventional mechanism of action of aminopyridines (including 3,4-DAP). These studies suggest that aminopyridine-induced increases in neurotransmission associated with the treatment of LEMS and other neurological diseases may result from an alternative agonistic mechanism of action on Cav1 type (also called "L-type")

voltage-gated calcium channels, resulting in greater calcium influx into the nerve terminal (Li et al., 2014; Wu et al., 2009). However, the relevance of the reported effects is debated because the 3,4-DAP concentrations used in these more recent studies were significantly above those used in LEMS treatment conditions measured in serum after typical dosing (Meriney & Lacomis, 2018; Wu et al., 2018). Furthermore, because Cav1 channels usually lack the synaptic protein interaction sites present in Cav2 channels (Catterall, 1999; Mochida et al., 2003; Sheng, Westenbroek, & Catterall, 1998), Cav1 channels are thought to reside outside of synaptic vesicle release sites in the neuromuscular junction and therefore are not thought to directly control acetylcholine release (as Cav2 channels do) at healthy adult synapses. However, it is possible that Cav1 channels may have a minor role at neuromuscular synapses that is revealed under variable pharmacological conditions (Atchison & O'Leary, 1987; Pancrazio, Viglione, & Kim, 1989; Atchison, 1989; Flink & Atchison, 2003; Urbano, Rosato-Siri, & Uchitel, 2002) and Cav1 channels may have a compensatory contribution to the control of transmitter release in diseased conditions such as LEMS (Flink & Atchison, 2002; Xu, Hewett, & Atchison, 1998).

Therefore, to investigate the physiological mechanism accounting for the clinical response to 3,4-DAP, we tested the effects of therapeutic (1.5 μM) concentrations of 3,4-DAP on Kv3 potassium channels expressed in tSA-201 cells, evaluated how 3,4-DAP affects the presynaptic AP waveform at motor nerve terminals, and tested the effects of 3,4-DAP on transmitter release from weakened frog and mouse NMJs. To explore the role of Cav1 channels in 3,4-DAP-mediated effects at NMJs, we conducted our experiments in the presence and absence of a Cav1 antagonist (nitrendipine). In addition, we examined effects of a supra-therapeutic concentration of 3,4-DAP (100 μM) to allow direct comparisons with some prior studies (Li et al., 2014; Wu et al., 2009; Wu, Chen, & Pan, 2018). For the purposes of this report, we define supra-therapeutic as

concentrations that are about 100 fold higher than the measured concentrations in the serum of LEMS patients after taking the typical prescribed dose of 3,4-DAP (Haroldsen et al., 2015a; Haroldsen et al., 2015b; Ishida et al., 2015; Thakkar et al., 2017; Wirtz et al., 2009). Our results indicate that 1.5 μM 3,4-DAP has a small but significant effect selectively on Kv3.3 channels, and that it broadens the presynaptic AP and increases neuromuscular transmission independent of a Cav1 contribution. The effects of the supra-therapeutic concentration of 100 μM 3,4-DAP were much greater, but remained independent of a Cav1 contribution. These results favor the hypothesis that the clinical effects of 3,4-DAP in the treatment of LEMS are caused by a partial block of voltage-gated potassium channels independent of any effects of Cav1 voltage-gated calcium channels.

2.2 Methods

2.2.1 Ethics statement

The experimental procedures in this study were conducted in compliance with the US National Institutes of Health laboratory animal care guidelines and approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. All efforts were made to minimize the suffering of animals.

2.2.2 Tissue preparation

Adult frogs (rana pipiens) were anaesthetized via immersion in 0.6% tricaine methane sulphonate, decapitated and double pithed. The *ex vivo* cutaneous pectoris neuromuscular preparation was dissected and bathed in normal frog Ringer saline (NFR, in mM: 116 NaCl, 10 mM BES buffer, 2 mM KCL, 5 mM glucose, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.3). Adult Swiss Webster mice (3-6 months of age; Charles River Laboratories, Wilmington, MA) were sacrificed using CO2 inhalation, followed by thoracotomy. The epitrochleoanconeous neuromuscular preparation was bilaterally dissected out and bathed in normal mouse Ringer saline (NMR, in mM: 150 NaCl, 10 mM BES buffer, 5 mM KCL, 11 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4).

2.2.3 Intracellular microelectrode electrophysiology

The muscle nerve was stimulated using a suction electrode and muscle contraction was blocked following a 1 hr incubation in a bath containing 50 μM of the irreversible muscle myosin inhibitor BHC (Heredia, Schubert, Maligireddy, Hennig, & Gould, 2016). After BHC washout using normal saline, microelectrode recordings were made in the presence of 1 μM nitrendipine (Sigma, St. Louis, MO) or the vehicle, 0.01% DMSO, plus a selective muscle voltage-gated sodium channel blocker (1 μM μ-conotoxin PIIIA for the frog NMJ or 5 μM μ-conotoxin GIIIB for the mouse NMJ; Alomone Labs Ltd, Jerusalem, Israel). Additionally, in order to reduce the magnitude of transmitter released (to maintain accuracy of corrections for non-linear summation during analysis), 250-900 nM ω-conotoxin GVIA (to block N-type channels at the frog NMJ) or 50 – 100 nM ω-agatoxin GIVA (to block P/Q-type channels at the mouse) was included in the recording bath. The range of concentrations listed was used iteratively with each preparation to

reduce control endplate potentials (EPPs) to below 10 mV. Intracellular recordings of muscle cell membrane potentials were obtained using borosilicate glass microelectrodes pulled to a resistance of ~40-60 MΩ and filled with 3 M potassium chloride. For each muscle fiber recording made adjacent to visualized NMJs, spontaneous miniature synaptic events (mEPPs) were collected for 1-2 min, followed by 10-30 EPPs elicited by low frequency (0.2 Hz) stimulation with an interstimulus interval of 10 s. Subsequently, neuromuscular preparations were incubated in freshly made 1.5 µM or 100 µM 3,4-DAP for 30-60 minutes. After 3,4-DAP incubation, paired recordings were made from the same neuromuscular junctions that had been studied in control saline (resulting in paired data sets). Data were collected using an Axoclamp 900A and digitized at 10 kHz for analysis using pClamp 10 software (Molecular Devices). Spontaneous and evoked membrane potentials were normalized to -70 mV and corrected for non-linear summation (McLachlan & Martin, 1981). We measured the magnitude of transmitter release by determining the quantal content (QC) using the direct method of dividing the peak of the averaged EPP trace by the peak of the averaged mEPP trace.

2.2.4 Voltage imaging

Recordings were performed as described previously (Ginebaugh et al., 2020). Voltage imaging was performed on the cutaneous pectoris nerve-muscle preparations from both male and female adult frogs (*Rana pipiens*), as well as the ETA nerve muscle preparation from both male and female adult mice. To load nerve terminals with dye for the voltage-imaging procedure, a mixture of 5 mL of NFR (for frog preparations) or NMR (for mouse preparations) with a BeRST 1 voltage-sensitive dye (Huang, Walker, & Miller, 2015) concentration of 0.5 µM and 10 ug/mL

of Alexa Fluor 488-conjugated α-bungarotoxin (BTX; to counterstain postsynaptic receptors at the NMJ and block muscle contractions) was mixed fresh daily. Then, the neuromuscular preparation was bathed in this dye mixture for 90 minutes, rinsed, and mounted on the stage of an Olympus BX61 microscope with a 60x water immersion objection. The nerve was then drawn into a suction electrode for supra-threshold stimulation. If the BTX conjugated to Alexa Fluor 488 did not completely block muscle contractions, 10 μM of curare was added to the imaging saline to completely block any remaining nerve-evoked muscle contractions.

The postsynaptic BTX stain was used to identify nerve terminals and bring them into focus for voltage imaging. After locating a well-stained nerve terminal, an imaging region of interest (ROI) that contained a large portion of the nerve terminal branch (usually an ROI of approximately 80x30 μm for frog or 60x40 μm for mouse) was selected. All voltage imaging recordings were performed at room temperature (20-25 °C).

After a nerve terminal was selected for imaging, the presynaptic nerve was stimulated at 0.2 Hz. During each stimulation, there was a brief 100 μs image collection window where the preparation was illuminated by a 640 nm laser (89 North laser diode illuminator) and the BeRST 1 dye fluorescence of the nerve terminal was recorded by an EMCCD camera (Pro-EM 512, Princeton Instruments). A custom routine on a Teensy 3.5 USB development board (PJRC) created a delay between the stimulation of the nerve and the triggering of the camera and laser. This delay in the 100 us collection window was increased by 20 μs after each stimulation, and after 100 sequential delays of 20 μs the delay a full time course of 2 ms was obtained (for 100 μM 3,4-DAP recordings, 300 moving bins were recorded for a total time course of 6 ms). Using this moving-bin acquisition scheme, where 100 or 300 bins were collected sequentially over a 2 or 3 ms time course, the entire AP waveform was sampled. For each frog nerve terminal recording, this process

was repeated 5-15 times, and resulting AP waveforms were averaged. For mouse nerve terminal recordings, this process was repeated 10-50 times (the BeRST 1 dye signal at the mouse terminals was weaker than at the frog terminals, and thus required more recordings to obtain a high quality averaged AP waveform).

Custom written scripts in ImageJ and MATLAB (Mathworks) were used to analyze images. An "align slices in stack" ImageJ plugin (https://sites.google.com/site/qingzongtseng/template-matching-ij-plugin; see (Q. Tseng et al., 2012) was used to stabilize the image stack for x-y drift. Then, an unbiased ROI selection (a subsection of the full imaging ROI) containing the nerve terminal was created by applying an Otsu local imaging threshold (Otsu, 1979) to the average fluorescence z-projection of the stabilized BeRST 1 image stack. The average fluorescence inside this ROI was used as the nerve signal (for frog recordings, 20 μm near the end of the nerve terminal and last node of Ranvier were excluded to restrict recordings to the middle electrical region of the terminal; see (Ginebaugh et al., 2020). The average fluorescence from the region outside of the Otsu selected ROI was used as the background signal. Both the nerve and background signals were then low-pass filtered offline (f_{pass}= 4 kHz). The following analysis was then performed separately for both the filtered and unfiltered signals: the background signal was divided from the nerve signal to give us a fluorescence signal. We then fit a cubic B-spline through the unstimulated points in the fluorescence time course (the first and last 15 points of the 100 total points in each time series), and divided this cubic spline from each point in the fluorescence signal. This gave us a $\Delta F/F$ fluorescence signal that did not fluctuate as a result of drift of the nerve muscle preparation or dye bleaching.

Two AP waveforms were then created by separately averaging the APs from the filtered and unfiltered ΔF/F fluorescence signal. The R^2 value between the filtered and unfiltered AP waveforms was then calculated. Since these are not linear models, the R^2 is not an exact measure of fit between the filtered and unfiltered AP waveforms, but rather is a metric of fit that is heavily weighted by the strength (in terms of the amplitude of the ΔF/F fluorescence signal) of the recording. This weight is important because normalizing slight bumps on an almost flat signal could appear as an AP. Thus, the R^2 value provides a heuristic metric to estimate the quality of our recorded AP waveforms, and is not used for any statistical purposes.

Image artifacts in the background (e.g. a BeRST 1 dye stained free-floating piece of connective tissue) occasionally resulted in the background, resulting in a noisier signal (and worse R^2 value). If the R^2 was less than 0.95 for frog recordings (or 0.90 for the 300 bin recordings for the 100 μM DAP recordings), or 0.85 for the mouse recordings, the fluorescence of an approximately 15x30 μm section of the background near the nerve terminal was used as the background fluorescence rather than the complete background region. If this smaller background subsection also resulted in a R^2 value higher than the values listed above, the recording was not included in the data analysis. If the recording was of high enough quality to produce a sufficient R^2 value the average filtered AP was normalized to the first 15 points (the baseline of the trace) and fit with cubic spline interpolation at an oversampled time resolution of 2 μs. Finally, the full width at half maximum (FWHM) of the AP waveform was calculated. Further details of this method are presented in (Ginebaugh et al., 2020).

2.2.5 Whole-cell perforated patch clamp electrophysiology

Recordings were performed as previously described (Wu et al, 2018), using tSA-201 cells

transfected with Kv3.3 (α subunits), or Kv3.4 (α subunits). All recordings were performed at room temperature (20-22 °C). Cells were bathed in a saline containing 130 mM NaCl, 10 mM Hepes, 10 mM glucose, 3 mM $CaCl_2$, and 1 mM $MgCl_2$ at pH 7.4. The pipette solution contained 70 mM K_2SO_4, 60 mM KCl, 10 mM Hepes, and 1 mM $MgCl_2$ at pH 7.4. Patch pipettes were fabricated from borosilicate glass and pulled to a resistance of ~1-4 MΩ. Before each experiment, 3 mg amphotericin-B was dissolved in 50 μL DMSO. Approximately every hour, 20 μL of this stock amphotericin solution was mixed with 1 mL filtered pipette solution. Pipettes were filled in a two step process. The tip was dipped into a droplet of filtered pipette solution for 1-3 s, and then the remainder of the pipette was back-filled with the amphotericin-B/pipette solution mixture using a syringe and a 34 G quartz needle (MicroFil MF34G, World Precision Instruments, Sarasota, FL). This pipette was then used to make a GΩ seal with a fluorescent cell and given time for the amphotericin-B to perforate the patch of membrane isolated under the pipet in order to gain electrical access to the whole cell (5-10 min). Access resistances ranged from 10 to 20 MΩ and were compensated by 80-85%. All chemicals were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless otherwise noted. Voltage clamp of cells was controlled by an Axopatch 200B amplifier driven by Clampex 9 or 10 software (Molecular Devices, Sunnyvale, CA). Data were filtered at 5 kHz and digitized at 10 or 50 kHz. Data were analyzed using Clampfit 10 software (Molecular Devices). Capacitive transients and passive membrane responses to the voltage steps were subtracted, and a liquid junction potential of -3.6 mV was corrected before each recording. Current through calcium channels was activated by depolarizing steps from a holding potential of -80 mV to +40 for 500 msec before returning to -80 mV for evaluation of Kv3.3 or Kv4.4 current amplitude before and after application of 3,4-DAP. These depolarizing steps were given every 5-30 s to allow Kv channels to recover between stimuli. 3,4-DAP was dissolved in saline at 5 M to

create a stock solution on the day of use and used at the concentrations (1.5, 100, 500, 5000 μM) indicated for application to cells. Drug solutions were delivered directly to a cell under study using a pressurized borosilicate glass pipette lowered over the cell during the recording.

2.2.6 Statistical analysis

Data were statistically analyzed using SPSS Statistics v.25 (IBM) and Prism v.7 (Graphpad). Data were determined to be outliers if the data exceeded 1.5 times the interquartile range. Distribution was assessed for normality using Shapiro-Wilk test (for $n \leq 50$) or Kolmogorov-Smirnov test (for $n > 50$). Statistical comparisons were performed using a mixed factorial ANOVA with between-subjects factor of bath (nitrendipine or control bath) and within-subjects factor of time (pre or post 3,4-DAP), with Tukey's post-hoc comparisons when appropriate. For comparisons of measurements within a single manipulation (e.g. before and after 3,4-DAP application), we used Student's *t-test* (if distribution was normal) or Wilcoxon matched-pairs signed rank test (if distribution was not normal). Results were considered statistically different when the *p*-value was <0.05. The results represent the ± s.e.m. of at least three independent experiments.

2.3 Results

2.3.1 3,4-DAP dose-dependently increases neuromuscular transmission independent of Cav1 channels

To characterize the dose-dependent effects of 3,4-DAP at the neuromuscular junction, we used paired intracellular microelectrode recordings in *ex vivo* neuromuscular preparations to measure endplate potentials (EPPs) in response to nerve-evoked APs in *ex vivo* neuromuscular preparations both before and after exposure to either low or high micromolar concentrations of 3,4-DAP. Additionally, we measured spontaneous miniature EPPs (mEPPs) from the same population of muscle fibers to determine quantal content. We performed these recordings in the presence or absence of the Cav1 blocker nitrendipine to test the hypothesis that these L-type calcium channels are important for 3,4-DAP effects.

To reduce the magnitude of transmitter release, we performed all recordings in the presence of low doses of Cav2.1 (ω-agatoxin IVA for mouse NMJs) or Cav2.2 (ω-conotoxin GVIA for frog NMJs) calcium channel antagonists. Reducing transmitter release magnitude mimics the effects of neuromuscular diseases, minimizes complications due to non-linear summation, and ensures that corrections for non-linear summation are accurate after 3,4-DAP application. In the absence of these selective Cav2 calcium channel blockers, control EPPs average 10-40 mV in amplitude above resting membrane potential (e.g. from -70 mV resting membrane potential to a peak of -60 to -30 mV) at the mouse and frog NMJs (Sosa & Zengel, 1993; Tarr, Malick, et al., 2013). Therefore, after exposure to 3,4-DAP (especially the 100 μM concentration), EPPs can increase to very near the acetylcholine receptor channel reversal potential (-10 mV), making 3,4-DAP-induced changes in transmitter release difficult to analyze. For these reasons, all experiments were

performed using a calcium channel blocker to reduce quantal content and allow a more accurate measurement of 3,4-DAP effects.

We first evaluated the effects of 3,4-DAP at the mouse NMJ (Fig. 11), since mouse NMJs are phylogenetically similar in structure and calcium channel subtype expression to human NMJs. After exposure to ω-agatoxin IVA, a mean mEPP amplitude of 0.4 ± 0.04 mV, and nerve stimulation produced a mean EPP of 5.2 ± 0.3 mV, and. Using these values to calculate the quantal content (QC) from these synapses (a measure of the number of vesicles released; QC = corrected mean EPP / corrected mean mEPP amplitude), motoneuron APs on average elicited a release of approximately 14 quanta per trial (14.2 ± 1.2; Fig. 11E, F). Bath application of a clinically relevant dose of 3,4-DAP (1.5 μM) increased EPP amplitudes approximately 3.2-fold (16.3 ± 0.9 mV), without altering mEPP amplitudes (0.4 ± 0.03 mV), increasing quantal content to approximately 44 quanta per trial (44.1 ± 2.6; a 3.1-fold increase). These effects are similar to 3,4-DAP effects reported previously in LEMS model mice (Tarr et al., 2014; Tarr, et al., 2013). Next, we next evaluated the effects of a supra-therapeutic concentration of 3,4-DAP (100 μM) in 3,4-DAP-naïve preparations. After exposure to ω-agatoxin IVA, motoneuron APs produced a mean baseline EPP amplitude of 5.8 ± 0.3 mV, with a mean mEPP amplitude of 0.5 ± 0.02 mV. Quantal release per trial prior to treatment was approximately 16 (15.5 ± 1.2; Figure 11H, I). After 100 μM 3,4-DAP application, the mean EPP amplitude was increased by approximately 9.4-fold (50.8 ± 2.1 mV), without a significant change in mean mEPP amplitude (0.4 ± 0.02 mV). Quantal content was increased from 15.5 quanta per trial to 126.3 ± 6.1 mV, an 8.2-fold increase in quantal release.

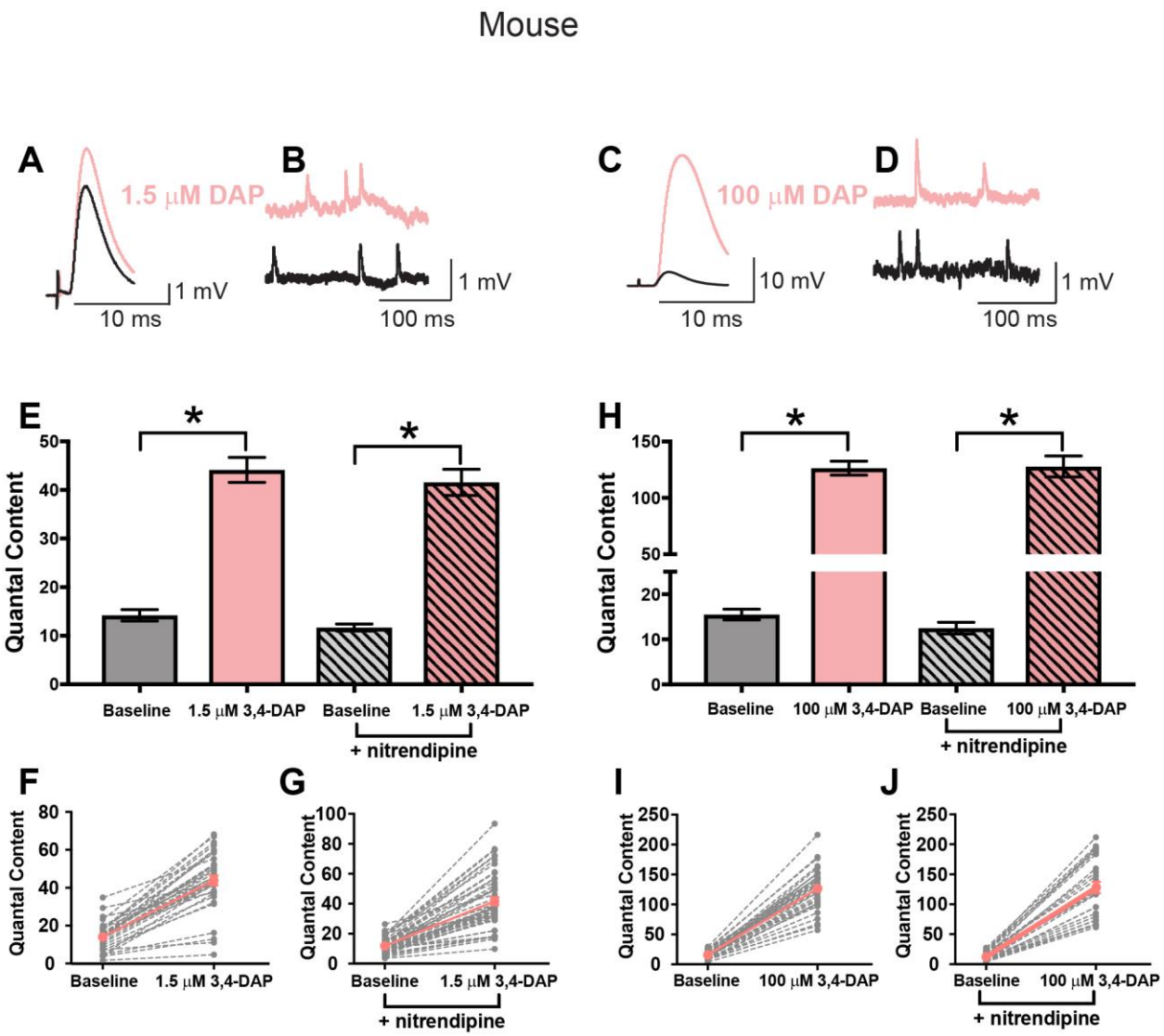

Figure 11 3,4-DAP dose-dependently increases neuromuscular transmission independent of Cav1 channels in mouse neuromuscular junctions.

(A-D) Sample traces of electrophysiological recordings before and after 1.5 μM (A-B) or 100 μM (C-D) 3,4-DAP application. (E) 1.5 μM 3,4-DAP significantly increases quantal content (solid bars) independent of nitrendipine (striped bars). Two-way ANOVA with Tukey's post hoc analysis, * $p < 0.0001$. (F-G) Individual paired recordings of quantal content pre- and post-3,4-DAP, with or without nitrendipine, are shown (grey dotted lines), with a superimposed average (solid pink line). Student's t-test, * $p < 0.0001$. (H) 100 μM 3,4-DAP significantly increases quantal content (solid bars) independent of nitrendipine (striped bars). (I-J) Individual paired recordings of pre and

post quantal content, with or without nitrendipine, is shown (grey dotted lines), with a superimposed average (solid pink line). Student's t-test (I) and Wilcoxon matched-pairs signed rank test (J), *** $p < 0.0001$.

Because it has been reported previously that millimolar doses of 3,4-DAP increase neurotransmission via augmented Cav1 conductance, we evaluated this hypothesis by using the Cav1 antagonist nitrendipine to determine if blocking Cav1 channels would alter the effects of 1.5 or 100 μM 3,4-DAP. After ω-Agatoxin block in the presence of nitrendipine, EPP amplitudes averaged 4.8 ± 0.3 mV, and mean mEPP amplitude was 0.4 ± 0.02 mV (QC = 11.7 ± 0.7; Fig. 11E, G). After application of 1.5 μM 3,4-DAP, mean EPP amplitude increased about 3.7-fold (41.6 ± 2.7 mV), without altering mEPP amplitude (0.4 ± 0.02), and quantal content increased 3.5-fold. This increase in vesicle release caused by 1.5 μM 3,4-DAP in mouse NMJs was not significantly different from our recordings without nitrendipine (Fig. 11E), indicating that antagonism of Cav1 channels did not alter the effects of a clinically-relevant concentration (1.5 μM) of 3,4-DAP at the mouse neuromuscular junction. We next assessed whether nitrendipine could modulate the effects of a supra-therapeutic dose of 3,4-DAP (100 μM). After ω-Agatoxin treatment in the presence of nitrendipine, EPP amplitude averaged 5.7 ± 0.4 mV, and mEPP amplitude averaged 0.4 ± 0.02 mV (QC = 12.5 ± 1.3; Fig. 11H, J). Bath application of 100 μM 3,4-DAP increased EPP amplitude by about 13.5-fold (70.5 ± 3.4 mV), and mEPP amplitude was not significantly changed (0.5 ± 0.04 mV). Quantal content increased to 127.8 ± 9.4 after application of 100 μM 3,4-DAP, a 10.2-fold increase, and nitrendipine did not alter the effects of 100 μM 3,4-DAP (Fig. 11H).

We also tested the effects of 3,4-DAP at the frog neuromuscular junction, a traditional model of neuromuscular function. After exposure to ω-conotoxin GVIA, APs produced a mean EPP amplitude of 3.7 ± 0.6 mV, and spontaneous release resulted in a mean mEPP amplitude of 0.8 ± 0.07 (QC = 4.8 ± 1.4; Fig. 12E, F). After application of 1.5 μM 3,4-DAP, the mean EPP

amplitude increased approximately 2-fold (to 7.6 ± 1.4 mV), without significantly altering mEPP amplitude (0.9 ± 0.1). After exposure to 1.5 μM 3,4-DAP, quantal content increased to 8.77 ± 1.5 (a 1.8-fold increase). Subsequently, we assessed the effects of the supra-therapeutic concentration of 100 μM 3,4-DAP. Before DAP exposure, motoneuron APs produced an EPP amplitude of 2.6 ± 0.4 mV, and spontaneous release resulted in a mean mEPP amplitude of 0.7 ± 0.07 mV (QC = 3.8 ± 0.6; Fig. 12H, I). After 100 μM 3,4-DAP treatment, the EPP amplitude increased to an average of 93.7 ± 10.7 mV, while mEPP amplitude did not significantly change (0.9 ± 0.09 mV). This resulted in an increase in quantal content to 142.6 ± 27 (a 37.5–fold increase).

We also evaluated the effects of a Cav1 blocker (nitrendipine) on 3,4-DAP effects at the frog neuromuscular junction. In the presence of nitrendipine baseline EPP amplitude was 1.3 ± 0.2 mV, and the mean mEPP amplitude was 0.5 ± 0.07mV (QC = 3.2 ± 0.5; Fig. 12E, G). After bath application of 1.5 μM 3,4-DAP in the presence of nitrendipine, the mean EPP amplitude increased by about 2.4-fold to 2.9 ± 0.5 mV, without significantly altering mEPP amplitudes (0.6 ± 0.08 mV), resulting in a quantal content of 6.0 ± 0.8 (a 1.9-fold increase). Therefore, nitrendipine did not significantly alter the effectiveness of 1.5 μM 3,4-DAP (Fig. 12E).

We next assessed whether nitrendipine could modulate 100 μM 3,4-DAP effects on neurotransmission at the frog neuromuscular junction. For these experiments, baseline, EPP amplitude in the presence of nitrendipine was 1.9 ± 0.4 mV, with an average mEPP amplitude of 0.8 ± 0.06 mV (QC = 2.9 ± 0.6; Fig. 12H, J). After bath application of 100 μM 3,4-DAP in the presence of nitrendipine, EPP amplitude increased to 54.9 ± 11.9 mV, while mEPP amplitude did not change significantly (0.9 ± 0.08 mV; QC = 67.8 ± 13.6, a 23.4–fold increase). There was no significant difference between the effect of 100 μM 3,4-DAP in the absence or presence of nitrendipine ($F(1, 19) = 4.162$, $p = 0.06$; Fig. 12H).

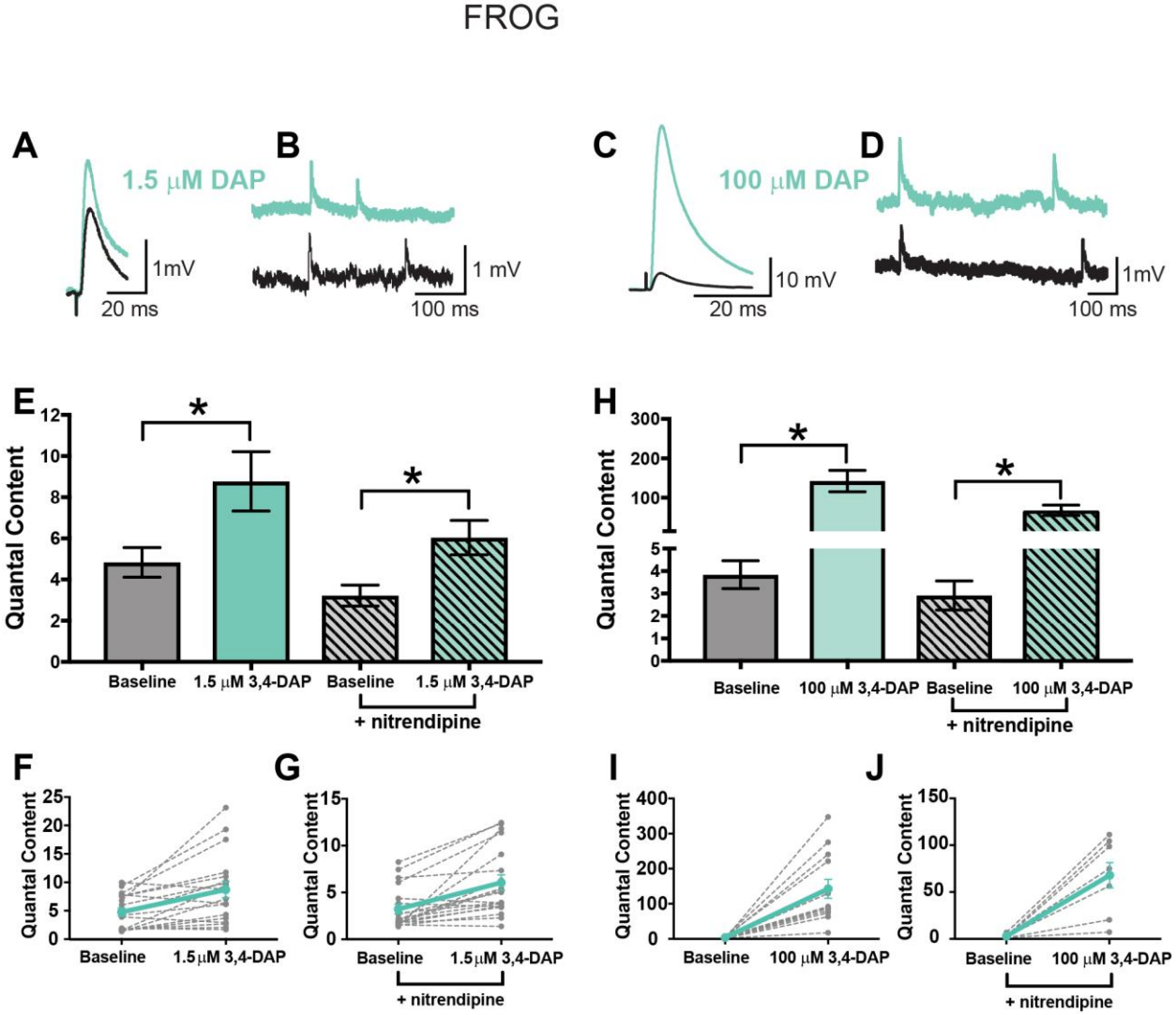

Figure 12 3,4-DAP dose-dependently increases neuromuscular transmission in frog neuromuscular junctions independent of Cav1 channels.

(A-D) Sample traces of electrophysiological recordings before and after 1.5 μM (A-B) or 100 μM (C-D) 3,4-DAP application. (E) 1.5 μM 3,4-DAP significantly increases quantal content (solid bars) independent of nitrendipine (striped bars). (F-G) Individual paired recordings of quantal content pre- and post-3,4-DAP, with or without nitrendipine, are shown (grey dotted lines), with a superimposed average (solid pink line). Wilcoxon matched-pairs signed rank test (F) and Student's t-test (G), * $p < 0.005$. (H) 100 μM 3,4-DAP significantly increases quantal content (solid bars) independent of nitrendipine (striped bars). (I-J) Individual paired recordings of pre and post quantal content, with or without nitrendipine, is shown (grey dotted lines), with a superimposed average (solid green line). Student's t-test (I) and Wilcoxon matched-pairs signed rank test (J), * $p < 0.05$..

2.3.2 3,4-DAP effects on the presynaptic AP waveform at the NMJ

The mechanism of action underlying the effects of 3,4-DAP on transmitter release at the NMJ is canonically thought to be due to broadening of the AP, but to date no studies have directly measured 3,4-DAP modulation of the AP waveform at the neuromuscular junction. Thus, we utilized a voltage-sensitive fluorescent dye (BeRST 1) to directly measure the impact of 3,4-DAP on the duration of the presynaptic AP waveform at NMJs. We performed a paired experiment where the AP waveform was recorded from a single terminal, the nerve-muscle preparation was loaded with 3,4-DAP, and the procedure was repeated on the same terminal. This was performed both with and without nitrendipine to test for an interaction between 3,4-DAP and Cav1 (L-type) voltage-gated calcium channels.

We first measured the impact of 1.5 μM DAP on the duration of the presynaptic AP waveform at mouse motor nerve terminals, and the interaction of nitrendipine with this effect (Fig. 13). We found a significant effect of 1.5 μM 3,4-DAP on the full width at half maximum (FWHM) of the presynaptic AP ($p = 0.001$, $n = 11$, mixed ANOVA), with the presynaptic AP FWHM increasing by 20.4% (53.95 ± 11.97 μs) after the addition of 1.5 μM 3,4-DAP. We found no effect of nitrendipine on the 3,4-DAP-induced change in duration of the AP waveform (Fig. 13E).

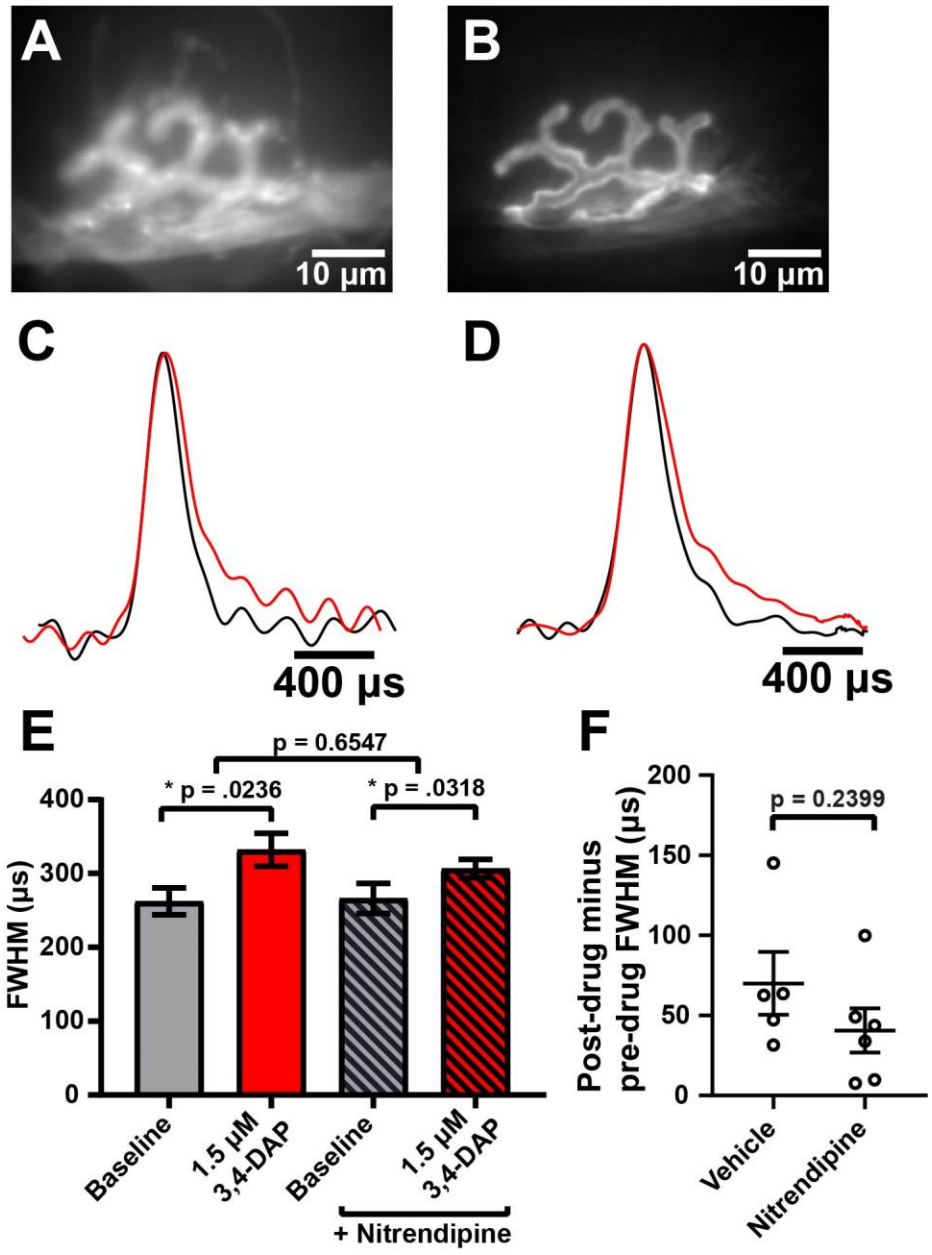

Figure 13 Therapeutic concentrations of DAP broaden the presynaptic AP waveform independent of Cav1 channels at the mammalian NMJ.

(A) A BeRST 1 dye stained image of a mammalian presynaptic motor nerve terminal. (B) An Alexa Fluor 488 α-BTX stained image of the same terminal as in (A). (C) Normalized presynaptic AP waveforms recorded from a nerve terminal before (black) and after (red) the addition of 1.5 µM DAP. (D) The normalized average of all pre-drug (black) and post-1.5 µM DAP (red) presynaptic AP waveforms recorded from mammalian motor nerve terminals (n = 11). (E) The presynaptic AP waveform is broadened by clinically relevant concentrations of 3,4-DAP both with

(striped bars) and without (solid bars) nitrendipine (paired t-test, $*p < 0.05$). The 3,4-DAP-induced broadening was independent of nitrendipine (mixed ANOVA, $p = 0.240$). Data collected by Scott Ginebaugh.

We then repeated the same experiment on the frog NMJ, a traditional model for the study of synaptic transmission (Fig. 14). Again, we found a significant effect of 1.5 μM DAP on the FWHM of the presynaptic AP ($p < 0.0001$, $n = 12$, mixed ANOVA), with the presynaptic AP FWHM increasing by 81.8% (225.16 ±25.23 μs) after the addition of 1.5 μM 3,4-DAP. Again, we found no interaction between nitrendipine and 3,4-DAP (Fig. 14E).

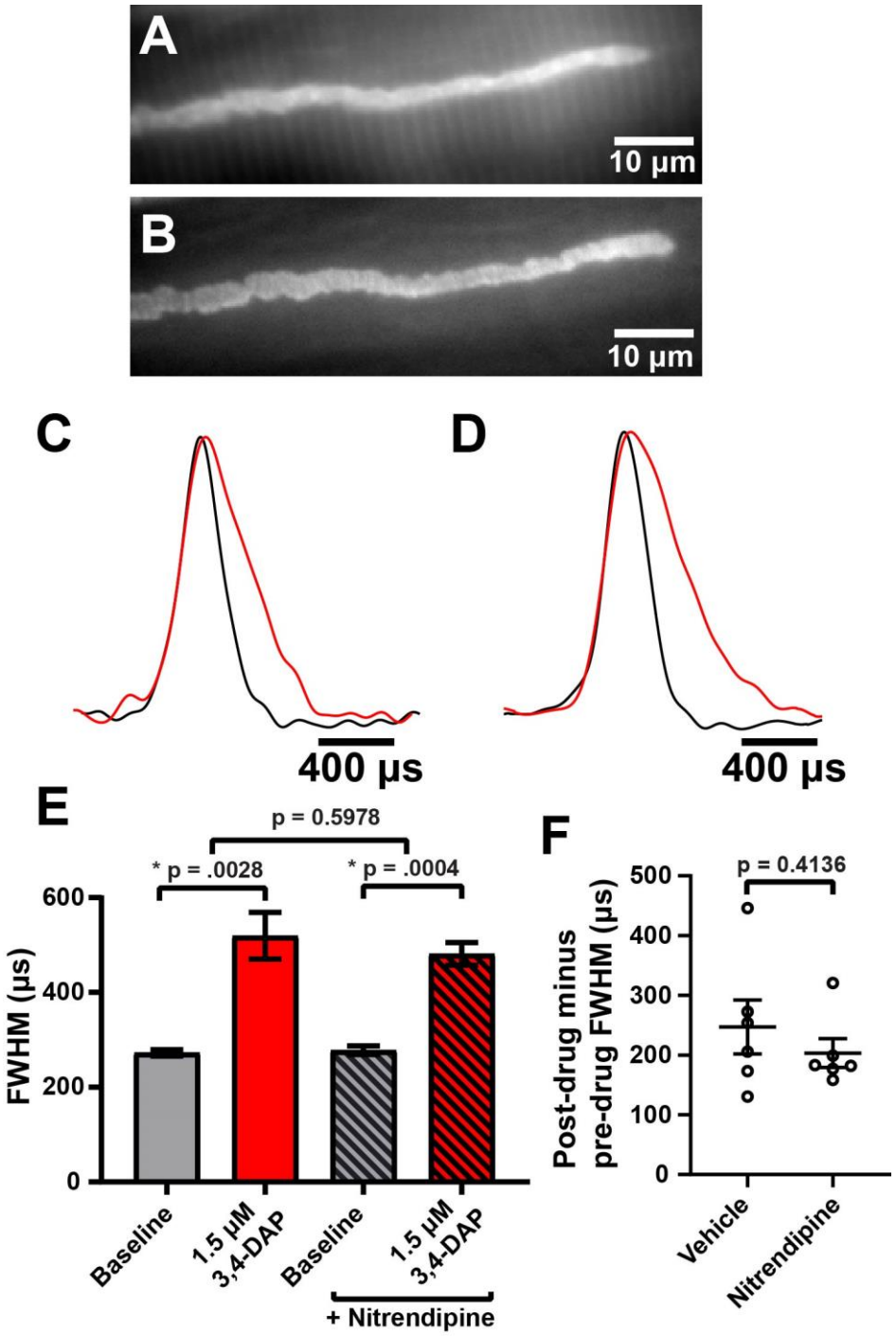

Figure 14 Therapeutic concentrations of DAP broaden the presynaptic AP waveform independent of Cav1 channels at the frog NMJ.

(A), A BeRST 1 dye stained image of a frog presynaptic motor nerve terminal. (B) an Alexa Fluor 488 α-BTX stained image of the same terminal as in (A). (C) Normalized presynaptic AP waveforms recorded from a nerve terminal before (black) and after (red) the addition of 1.5 µM DAP. (D) The normalized average of all pre-drug (black)

and post-1.5 μM DAP (red) presynaptic AP waveforms recorded from frog motor nerve terminals (n = 12). (E) The presynaptic AP waveform is broadened by clinically relevant concentrations of 3,4-DAP both with (striped bars) and without (solid bars) nitrendipine (paired t-test, *p < 0.005). The 3,4-DAP-induced broadening was independent of nitrendipine (mixed ANOVA, p = 0.414). Data collected by Scott Ginebaugh.

Finally, we tested the impact of a supra-therapeutic concentration (100 μM) of 3,4-DAP on the duration of the presynaptic AP waveform at the frog NMJ, and tested for an interaction of nitrendipine with the effect of this concentration of 3,4-DAP (Fig. 15). There was a significant effect of 100 μM DAP on the duration of the presynaptic AP waveform ($p < 0.0001$, $n = 9$, mixed ANOVA) with the AP FWHM increasing by 5.5 fold (1531.44 ± 209.11 μs). We did not see any significant interaction between nitrendipine and the effects of DAP on the presynaptic AP waveform at this supra-therapeutic concentration (Fig. 15D).

These data show that 3,4-DAP increases the duration of the presynaptic AP waveform at the NMJ in a dose-dependent manner, and that Cav1 (L-type) calcium channels have no interaction with this effect. Since small changes in the duration of the AP waveform can greatly increase calcium flux and transmitter release at the NMJ (Dodge & Rahamimoff, 1967; Ginebaugh et al., 2020), this magnified effect further supports the hypothesis that broadening of the AP via the blockage of voltage-gated potassium channels is the primary mechanism by which 3,4-DAP increases transmitter release *in vivo*.

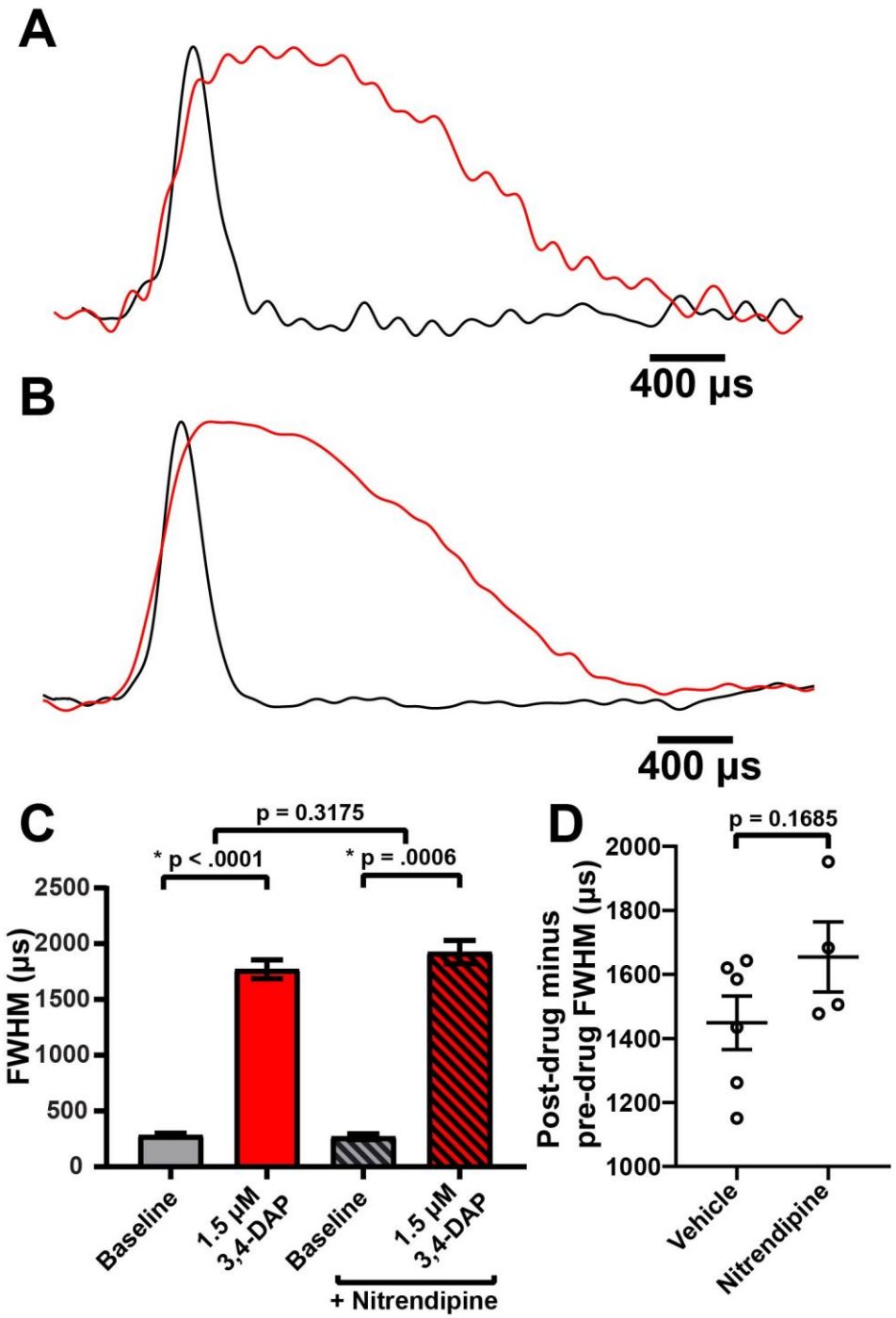

Figure 15 A supratherapeutic concentration of 100 µM DAP greatly broadens the frog presynaptic AP waveform independent of Cav1 channels.

(A) Normalized presynaptic AP waveforms recorded from a nerve terminal before (black) and after (red) the addition of 100 µM DAP. (B) The normalized average of all pre-drug (black) and post-100 µM DAP (red) presynaptic AP waveforms recorded form the frog motor nerve terminal (n = 10). (C) The presynaptic AP waveform is greatly

broadened by supra-therapeutic concentrations of 3,4-DAP both with (striped bars) and without (solid bars) the presence of nitrendipine (paired t-test, $*p < 0.05$). (D) The 3,4-DAP-induced broadening was independent of nitrendipine (mixed ANOVA, $p = 0.1685$). Data collected by Scott Ginebaugh.

2.3.3 3,4-DAP effects on Kv3 potassium channels

When considering which subtypes of Kv channel might be blocked by 3,4-DAP within mammalian motor nerve terminals, we were guided by prior work at the mouse NMJ which demonstrated that Kv3.3 and Kv3.4 were the types detected by immunohistochemistry (Brooke et al., 2004). To determine the effects of 3,4-DAP on these channel types, we expressed voltage-dependent Kv3.3 and Kv3.4 channels in tSA-201 cells and used whole-cell patch clamp electrophysiology to measure the change in current after 3,4-DAP application. Using a 500 ms step depolarization protocol (-100 mV to +40 mV), we activated Kv3.3 or Kv3.4 current and then measured the peak current before and after application of 3,4-DAP (at concentrations ranging between 0.10 μM to 5 mM). We found a concentration-dependent block of both Kv3.3 and Kv3.4 current that was very similar for each channel type. Importantly, the clinically relevant concentration of DAP (1.5 μM) significantly reduced Kv3.3 and Kv 3.4 current by about 10%; Figure 6). Curiously, we could not fit these data with the Hill equation. Instead, the data collected to date look like there might be two concentration response curves required to fit the data (one

high affinity and one low affinity) for 3,4-DAP. The exploration of this hypothesis will require collection of additional data.

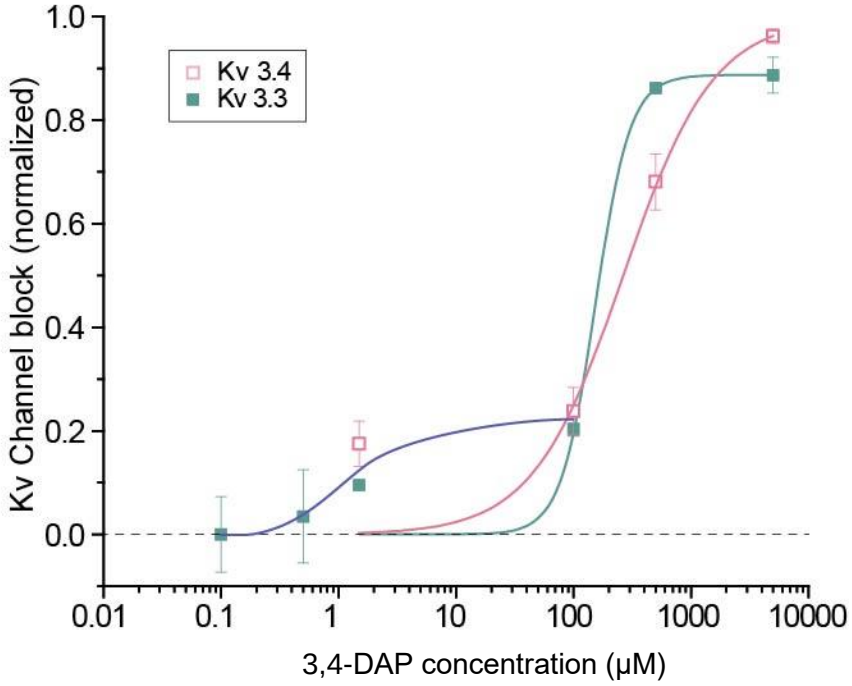

Figure 16 3,4-DAP effects on Kv3.3 and Kv3.4 potassium channels expressed in tSA-201 cells.

Preliminary 3,4-DAP concentration-inhibition data for Kv3.3 (solid teal squares) and Kv3.4 (open rose squares) channel antagonist activity concentration-response curves. Each current amplitude was normalized to its peak current amplitude before DAP application to derive the normalized block at each concentration. The teal and rose lines were fit with the Hill equation (Kv3.3, teal; Kv3.4, rose lines) and lead to the hypothesis that there might be a low affinity Kv binding site (IC50 = 150 μM for Kv3.3, and 250 μM for Kv 3.4, although these data are preliminary), while an additional line (blue line for both Kv3.3 and Kv3.4) indicates a hypothesized high affinity binding site (which requires more data to draw firm conclusions). Data collected by Man Wu.

2.4 Discussion

3,4-DAP is the FDA-approved first-line treatment for patients with Lambert-Eaton Myasthenic Syndrome (LEMS; (FDA press release, 2019; FDA press release, 2018; Voelker, 2019); a disease characterized by reduced neuromuscular transmission and subsequent muscle weakness (Lambert et al., 1956; Lang et al., 1984). 3,4-DAP has been shown to be only modestly beneficial in LEMS due to dose-dependent side effects that limit prescribed dosing in patients (Lindquist & Stangel, 2011; Wirtz et al., 2009) (Oh, 2016; Tarr, Wipf, & Meriney, 2015). Although 3,4-DAP is canonically thought to mediate its effects by partially blocking presynaptic voltage-gated potassium channels, a previous report that millimolar concentrations of 3,4-DAP could have off-target effects on Cav1 channels led to the question of whether 3,4-DAP effects at therapeutic concentrations involve Cav1 channels. For this reason, we sought to characterize the effects of 1.5 and 100 μM 3,4-DAP on transmitter release, the presynaptic AP waveform, Kv3 potassium channels, and to determine if the effects were altered by blocking Cav1 (L-type) calcium channels using nitrendipine. We found that the effects of 3,4-DAP at micromolar concentrations were explained completely by block of potassium channels and the resulting broadening of the presynaptic AP, independent of any contribution from Cav1 calcium channels.

In particular, we report a concentration-dependent effect of 3,4-DAP on the presynaptic AP waveform duration at the mouse and frog NMJ. AP repolarization is mediated by voltage-gated potassium channels, and at the mammalian presynaptic neuromuscular nerve terminal, potassium channel isoforms have been reported to be Kv3.3 and Kv3.4 (Brooke et al., 2004). Kv3 channels have been shown to have rapid activation and inactivation characteristics that enable nerve terminals to fire APs with short duration and at high frequency (Kaczmarek & Zhang, 2017; Zemel

et al., 2018), consistent with the AP waveforms recently optically recorded from frog and mouse presynaptic nerve terminals (Ginebaugh et al., 2020).

Here, we show that Kv3.3 and Kv3.4 channels are approximately equally blocked by 10% following exposure to the therapeutically relevant concentration of 1.5 μM 3,4-DAP. Previously, 4-aminopyridine has been reported to have IC50 values for blocking Kv3 channels of 30-1200 μM (depending in the Kv3 subtype; (Grissmer et al., 1994; Rettig et al., 1992; Vega-Saenz de Miera et al., 1992; Luneau et al., 1991; Muqeem et al., 2018), but we are not aware of any prior study characterizing the effects of 3,4-DAP on Kv3 channels. We observed a 10% decrease in Kv3.3 and Kv3.4 currents after 1.5 μM 3,4-DAP application, which we predict would have a significant effect on the AP duration in motor nerve terminals, since Kv3 currents have been shown to mediate the dominant outward current during brief AP depolarizations within other nerve terminals (Alle, Kubota, & Geiger, 2011). Furthermore, even relatively small changes in presynaptic AP duration (15-20%) have been predicted to have significant effects on calcium ion entry and transmitter release (Ginebaugh et al., 2020). Thus the roughly 20% broadening reported here at the mouse NMJ would be predicted to underlie the approximate 3-fold increase in transmitter release we observed (Ginebaugh et al., 2020). Interestingly, we found that 1.5 μM 3,4-DAP broadened the presynaptic AP waveform in the frog NMJ to a greater extent than at the mouse NMJ. However, a species difference in presynaptic ion channel expression that leads to these results is currently unknown.

At neuromuscular synapses, the very brief presynaptic AP waveform only activates a small percentage of the Cav2 voltage-gated calcium channels positioned within transmitter release sites (Luo et al., 2015; Luo et al., 2011). This is thought to ensure that each of the hundreds of transmitter release sites within each NMJ releases transmitter with low probability, conserving

resources for repeated activation during normal activity (Tarr et al., 2013). However, a brief AP activating only a small subset of available presynaptic Cav2 calcium channels leads to neuromuscular weakness after many of these calcium channels are attacked by autoantibodies in the disease LEMS. 3,4-DAP is an effective symptomatic treatment for LEMS because it broadens the presynaptic AP, increasing the percentage of available Cav2 calcium channels that open. A 3,4-DAP-mediated block of presynaptic Kv3 potassium channels broadens the presynaptic AP, which increases presynaptic calcium entry and transmitter release, leading to an improvement in neuromuscular strength in LEMS patients.

3.0 Targeting presynaptic calcium entry to improve neuromuscular transmission and motor function in spinal muscular atrophy mice

3.1 Introduction

Spinal muscular atrophy (SMA) is a lower α-motoneuron disease characterized by dysfunction and denervation of neuromuscular junctions (NMJs), atrophy of muscle fibers, and degeneration of motoneuron somata (Crawford & Pardo, 1996; Kariya et al., 2008; Kong et al., 2009; Lefebvre et al., 1995; Murray et al., 2008). The loss of motor units results from deleteriously low expression of a protein called Survival of Motor Neuron (SMN). As the name implies, SMN is integral to the survival of motoneurons and complete loss of this protein is lethal during embryogenesis (Gennarelli et al., 1995; Lefebvre et al., 1995; Monani, Sendtner, & Coovert, 2000). SMN is temporally regulated and elevated expression is rigidly aligned with the developmental window of neuromuscular maturation (Kariya et al., 2008; Gavrilina et al., 2008; Jablonka et al., 2000; Ling et al., 2012; Lutz et al., 2011). Loss of SMN results in extensive neuromuscular pathology, including moderate to severe reductions in the magnitude of transmitter release (quantified as quantal content) as well as the dissolution of neuromuscular synapses (Kong et al., 2009; Ling et al., 2010; Park et al., 2010; Ruiz et al., 2010; Tejero et al., 2016). In addition, individuals affected by SMA undergo a progressive loss of motor function (Swoboda et al., 2005). The severity of motor impairment comprises a broad phenotypic range, which can vary from minor changes in gait to paralysis and failure of respiratory function.

SMA is most often caused by the autosomal recessive inheritance of the *SMN1* gene with biallelic mutations or deletions (Lefebvre et al., 1995; Lefebvre et al., 1997; Wirth et al., 1997). In

humans, the majority of SMN derives from *SMN1* transcripts, and ~100% of translated protein from *SMN1* transcripts is full-length and functional (Lorson et al., 1999; Monani et al., 1999). Biallelic loss of *SMN1* usually results in a pathological reduction of SMN levels. Fortunately, humans uniquely express various copy numbers of a nearly identical homolog gene, *SMN2*. However, *SMN2* contains a crucial single nucleic polymorphism in exon 7, a mutation that appears in all individuals and reduces the efficiency of exon 7 inclusion during splicing (Lorson et al., 1999; Monani et al., 1999). Approximately 90% of *SMN2* transcripts lack exon 7, producing a truncated and unstable protein that is rapidly degraded. The presence of *SMN2* prevents embryonic lethality but often cannot produce enough functional SMN to prevent pathogenesis (Gennarelli et al., 1995; Lefebvre et al., 1995; Monani et al., 2000). Thus, *SMN2* copy number is a predominant modifier of disease severity and neuromuscular phenotype, with higher copy numbers inversely correlated with disease severity (Lorson et al., 1999) (Monani & Vivo, 2014). SMN is a ubiquitous protein found in all eukaryotic cells and performs various housekeeping roles, such as small nuclear ribonucleoprotein assembly, transcriptional regulation, and cellular trafficking (Singh et al., 2017). However, it remains unclear which disrupted role(s) of SMN causes motoneurons to be uniquely susceptible to extensive pathology.

The severe form of SMA is characterized by extensive denervation of neuromuscular junctions (NMJs), as well as neurotransmission abnormalities and morphological pathology (Boido & Vercelli, 2016; Cifuentes-Diaz et al., 2002; Kariya et al., 2008; Murray et al., 2008). NMJs are highly specialized structures that serve as the point of communication between the nervous and muscular systems. These synapses assemble near the center of muscle fibers and have specialized synaptic architecture and organization to develop a precisely apposed juxtaposition of presynaptic chemical transmitter release sites and postsynaptic receptors (Slater, 2015; Wu, Xiong,

& Mei, 2010). The sophisticated arrangement enables strong transmission that reliably brings the muscle fiber to threshold due to the large 'safety factor' that is characteristic of neuromuscular synapses (Wood & Slater, 2001). Under normal conditions, this safety factor ensures neurotransmission remains effective (initiating a postsynaptic action potential) despite changing physiological conditions and stresses, and is achieved by the release of excess acetylcholine. Neurotransmitter release is also a critical driver of NMJ development, as synaptic activity regulates the release and cleavage of key signaling molecules that drive NMJ maturation (Sanes & Lichtman, 1999b; Swenarchuk, 2019).

NMJ development is characterized by a change in postsynaptic NMJ receptor clusters (endplates), in which AChRs increase in density and are rearranged in their distribution. Endplates evolve from a uniform, oval-like plaque structure to an intricate pretzel-like structure, which is characterized by multi-perforated and invaginated structures and an enlarged circumference (Marques, Conchello, & Lichtman, 2000; Slater, 1982). Concurrently, motor nerve terminals undergo synapse elimination (from initially polyinnervated NMJs), radial growth and presynaptic differentiation to achieve mono-innervation of each endplate (Redfern, 1970; Sanes & Lichtman, 1999b). However, in SMA patients and SMA mouse model NMJs, this maturation is delayed; poly-innervation persists past normal developmental timeframes and endplates remain small and unperforated. Additionally, neurofilament, a nerve cytoarchitecture protein, accumulates in disorganized blebs at motor nerve terminals (Cifuentes-Diaz et al., 2002; Kariya et al., 2008; Murray et al., 2008).

A prominent feature in cellular and animal SMA models that may contribute to dysfunctional maturation caused by SMN deficiency is defective calcium homeostasis (Biondi et al., 2008; Jablonka et al., 2007; Lyon et al., 2014; McGivern et al., 2013; Ruiz et al., 2010; See et

al., 2013; Tejero et al., 2016). Voltage-gated calcium channels that regulate neurotransmitter release in motoneurons have a reduced expression in mouse models (Jablonka et al., 2007; Tejero et al., 2016), which corresponds with reduced calcium transients in motoneuron growth cones (Jablonka et al., 2007), neurotransmitter deficits at NMJs in mouse models (Kariya et al., 2008; Kong et al., 2009; Ling et al., 2010; Park et al., 2010; Ruiz et al., 2010; Tejero et al., 2016), axon elongation and guidance defects (Jablonka et al., 2007; Lyon et al., 2014; Rossoll et al., 2003), and delays in synaptic maturation (Kariya et al., 2008; Kong et al., 2009; Ruiz et al., 2010; Tejero et al., 2016).

Despite selective vulnerability to loss of SMN protein of the motor system, particular populations of motoneurons demonstrate resistance to denervation and degeneration (Ling et al., 2012). While the mechanism(s) underlying selective resistance remains elusive, it is evident that some motoneuron-muscle groups rapidly deteriorate in comparison to others (particularly those innervating head and trunk muscles, though some proximal and distal limb muscles are also vulnerable).

Recent advances in genetic therapies have proven miraculous in preventing the motoneuron degeneration caused by SMA (d'Ydewalle & Sumner, 2015). The first of these treatments uses postnatal administration of nusinersen, an *SMN2*-targeted antisense oligonucleotide (ASO) that blocks an intronic splice silencer in *SMN2* (ISS-N1), thereby increasing exon 7 inclusion in *SMN2* transcripts, which results in greater translation of full-length SMN (Rigo et al., 2014; Singh et al., 2006). Of the SMA patients (across the spectrum of severity types) treated with nusinersen, approximately half of them achieve significant improvements in motor function (Finkel et al., 2017; Mercuri et al., 2018). Remarkably, patients with 1-2 copies of *SMN2* (which usually constitute a severe phenotype) are able to achieve motor milestones that would have been

impossible without SMN-dependent therapy. However, due to the postnatal onset of clinical symptoms and extended time course of treatment required for serial ASO administrations, SMN upregulation begins after NMJ development is underway, and unlikely to fully rescue neuromuscular pathology particularly for patients with few *SMN2* copies or delayed intervention (Finkel et al., 2017; Mercuri et al., 2018). It is clear from mouse models that a critical and narrow therapeutic window for SMN-dependent therapy exists early in development during which high SMN levels are necessary to maximize the improvement of symptoms and mitigate neuromuscular pathology (Lutz et al., 2011; Murray et al., 2010; Bogdanik et al., 2015; Jablonka & Sendtner, 2017; Kariya et al., 2014; Le et al., 2011; Robbins et al., 2014; H. Zhou et al., 2015).

Clinical studies have shown that patients with *SMN1* deletions derive the greatest benefit to motor function and disease progression from early or presymptomatic ASO intervention (Finkel, et al., 2017; Mercuri et al., 2018). Despite this evidence, few countries neonatally screen for *SMN1* deletion and thus delay diagnosis until symptoms begin to manifest. Within the United States, several but not all states have adopted genetic screening for SMA at birth. Thus, SMN upregulation for patients with delayed ASO intervention may occur during or after the developmental stages requiring high SMN levels, resulting in irreversible pathology and dysfunction. Additionally, ASOs are administered to the central nervous system (via lumbar puncture) and may not fully disseminate to peripheral tissues (Bowerman et al., 2017), including NMJs. Deficits established during embryogenesis may not be reversible, particularly with late SMN-based therapeutic intervention, leaving NMJs vulnerable to deterioration later in life during normal aging or after injury. As such, the development of SMN-independent approaches to target neuromuscular function and improve muscle strength are necessary to improve the quality of life for SMA-affected individuals that experience limited benefits from SMN-dependent therapy.

We have evaluated a novel treatment that directly targets motor terminal calcium deficiency and supports neuromuscular function by augmenting action potential-driven calcium influx into synaptic vesicle release sites in motor nerve terminals. We tested the effect of GV-58, a positive allosteric calcium channel gating modifier, in combination with 3,4-DAP, a voltage-gated potassium channel antagonist. This drug combination has previously been established to synergistically increase neurotransmission in a mouse model of Lambert-Eaton myasthenic syndrome, a motoneuron disease characterized by reduced transmitter release caused by deficient motor terminal calcium (Tarr et al., 2014). Our results demonstrate that acute administration of GV-58 ± 3,4-DAP increases neuromuscular transmitter release and improves muscle strength in SMNΔ7 mice. Furthermore, we have shown that this new treatment can be used as a complement to ASO therapy to improve motor function.

3.2 Methods

3.2.1 Animal model

Mice of the strain "SMNΔ7" (FVB.Cg-Grm7Tg(SMN2)89Ahmb Smn1tm1Msd Tg (SMN2*delta7)4299Ahmb/J) were obtained from the Jackson Laboratory (stock number 005025). Male and female SMNΔ7 pups were raised and maintained in an animal breeding facility and maintained on a 14:10 light:dark cycle with *ad libitum* food and water in a humidity- and temperature-controlled room. All experiments were conducted in accordance with the policies set forth by University of Pittsburgh Institutional Animal Care and Use Committee and the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals. Litters from

heterozygote breeding pairs were randomly assigned into ASO or control groups, and drug treatment groups (in vivo or ex vivo behavior; vehicle, GV-58, or GV-58 + 3,4-DAP). Pups were tattooed for identification on postnatal day 0 (P0). Individual mice were genotyped from DNA obtained from tail snips by polymerase chain reaction.

3.2.2 ASO administration

For pups assigned to the ASO condition, P0-1 pups were cryo-anesthetized and receive a right cerebroventricular injection of 54 μg of the morpholino ISS-N1 [HSMN2Ex7D(-10,-29)] ASO including an opaque tracer (Evans Blue, 0.04%) (Porensky et al., 2012). Injections were performed as described by Porensky et al (2012) using a 30 gauge needle attached to a 10 ul syringe (Hamilton Company) guided by a transilluminator. Pups assigned to control treatment conditions did not receive an injection.

3.2.3 Drugs

For *ex vivo* treatments, GV-58 was dissolved in DMSO to achieve a working concentration of 50 uM in 0.1% DMSO in saline. 3,4-diaminopyridine was dissolved in saline to achieve a working concentration of 1.5 uM. For *in vivo* treatments, GV-58 was dissolved in the vehicle (2-propanol:PEG-300:TWEEN-80:water in a ratio of 13:24:39:11 (v/v)) and used at a dose of 150 mg/kg for subcutaneous administration. For treatments using GV-58 and 3,4-DAP, GV-58 was dissolved as described above with 3,4-DAP to achieve a working dose of 150 mg/kg of GV-58 and 0.07 mg/kg of 3,4-DAP. GV-58 doses were based on previous studies focused on the effects of

GV-58 on synaptic transmission in a motoneuron disease characterized by calcium influx dysfunction and low neurotransmission (Tarr et al., 2014). 3,4-DAP doses were based on previous studies focused on the effects of GV-58 + 3,4-DAP on synaptic transmission and the concentration of 3,4-DAP at neuromuscular junctions after administration of therapeutic doses (Tarr et al., 2014).

3.2.4 Behavioral testing

All behavior was conducted during the light cycle. For all behaviors, the average of 6 trials is reported, with a break of a minimum of 30 s between trials. Righting reflex and grip strength were recorded daily from P2-10. Pups were placed in a supine position on a quilted paper towel and the latency to stably upright (all 4 paws on the ground) was recorded for six trials and converted into a motor ability score using the following rubric: 0-2 s: 5; 3-5 s: 4; 6-10 s: 3; 11-20 s: 2; 21-30 s: 1; 31+ s: 0. Grip strength was measured as peak force in grams over a series of 6 trials. Pups were gently dragged across a circular wire mesh (7 cm) attached to a force gauge. *In vivo* drug administration in P10 pups was subcutaneously delivered between the shoulder blades, precluding the use of the righting reflex as a behavioral measurement. Baseline grip strength was measured and pups immediately received an injection of vehicle, GV-58, or GV-58 + 3,4-DAP. Grip strength was measured post-injection at 10 and 20 minutes to account for variable metabolism between pups. To control for variability in baseline grip strength within treatment groups (Genotype + ASO condition; + vehicle, GV-58, or GV-58 + 3,4-DAP), we set baseline grip strength as 100%, and report the greatest change from each pup's baseline after drug administration.

3.2.5 Intracellular recordings of neuromuscular junctions

Current clamp electrophysiology was performed on *ex vivo* epitrochleanconeus (ETA) and transverse abdominis (TVA) neuromuscular preparations of P11-13 mice. Mice were sacrificed by decapitation. Neuromuscular preparations were dissected out with the nerve branch(es) intact and bathed in normal mammalian Ringer (NMR, in mM: 150 NaCl, 10 mM BES buffer, 5 mM KCL, 11 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.3-7.4). Preparations were continuously gassed with 95% O2 and 5% CO2 and pinned over a bed of cured silicone rubber (Sylgard, Dow Corning Corp.) in a 5 ml chamber. Muscle contraction was blocked by a 1 hr incubation of an irreversible muscle myosin inhibitor, 50 μM BHC (Heredia et al., 2016). Prior to recording, the BHC was washed out. Microelectrode recordings were made in the presence of 2 μM μ-conotoxin GIIIB (to block skeletal muscle sodium channels) and either vehicle (0.1% DMSO; Sigma), GV-58 (50 uM), or GV-58 (50 uM) + 3,4-DAP (1.5 uM; Sigma) in saline. All recordings were performed after a 30 min incubation of vehicle or drug(s), and at room temperature (22 ºC). The nerve was stimulated using a suction electrode at 10x above threshold. Intracellular recordings of muscle cell membrane potentials were obtained using borosilicate glass microelectrodes pulled to a resistance of ~40-60 MΩ and backfilled with 3 M potassium chloride. Per fiber, spontaneous miniature synaptic events (mEPPs) were collected for 1-2 min, followed by 10-20 EPPs elicited by low frequency (0.2 Hz) stimulation with an interstimulus interval of 10 s. Data were collected using an Axoclamp 900A and digitized at 10 kHz for analysis using pClamp 10.7 software (Molecular Devices). Recordings were obtained from a minimum of 16 fibers per group and from a minimum of 3 animals. Spontaneous and evoked membrane potentials were normalized to -70 mV and corrected for non-linear summation (McLachlan & Martin, 1981) prior to analysis. The magnitude of transmitter release was measured as quantal content (QC). QC was directly calculated by dividing the peak of

the averaged EPP amplitude by the peak of the averaged mEPP amplitude (QC = EPP/mEPP).

3.2.6 Immunohistochemistry for neurofilament and maturation measurements

After dissection, ETA muscles were bathed in 1μg/ml α-bungarotoxin (Synaptic Systems) directly conjugated to Alexa-488 for 30 min to label postsynaptic acetylcholine receptors. Whole mount muscle tissue was fixed using 4% paraformaldehyde (w/v) in phosphate buffered saline (PBS) for 20 min at room temperature. The fixed tissue was then permeabilized and blocked using PBST (2% (w/v) bovine serum albumin, 2% (v/v) normal goat serum, and 0.5% Triton-X 100) for 20 min. The tissue was incubated overnight in a primary antibody to neurofilament medium chain (NF; mouse polyclonal, 1:500 dilution; Developmental Studies Hybridoma Bank, #NF-2H3) on a rocker at room temperature, and washed out the next day. Secondary antibody Alexa 647-conjugated goat-anti-rabbit (polyclonal, 1:1000 dilution, Invitrogen #A-21245) was incubated for 4 hr on a rocker at room temperature, then washed out. After a final PBS wash, whole tissue was mounted onto glass slides under #1.5 cover glass and using ProLong Gold mounting media (Thermo Fisher Scientific). Images were taken using a Leica TCS SP5 spectral confocal microscope using a 40x oil objective. Confocal scanning was performed using a resonance scanner at 8000 Hz and images were collected using line-scan averaging after 64 sweeps. Regions of interest were scanned using z-stacks of 0.5 - 1 μm steps to capture multiple synapses. Image analysis was performed using FIJI. Brightest projection images were made from stacks, and photon collections from both wavelengths were superimposed in pseudocolor to generate composite images. Only *en face* synapses were included in the analysis. For each genotype and treatment group, a minimum of 70 endplates were randomly selected from across the ETA muscle, and derived from at least 3 animals. Neurofilament signal overlapping α-BTX signal was measured per

synapse to determine neurofilament occupation of endplates.

3.2.7 Immunohistochemistry for synapse count and innervation

ETA muscles were dissected out, labeled for acetylcholine receptors, fixed, and washed as described above. Whole mount tissue was incubated overnight in primary antibodies to neurofilament (detailed above) and the synaptic vesicle marker SV2 (mouse monoclonal, 1:1000 dilution; Developmental Studies Hybridoma Bank, #SV2) on a rocker at room temperature, and washed out the next day. Secondary antibody Alexa 647-conjugated goat-anti-rabbit (polyclonal, 1:1000 dilution, Invitrogen #A-21245) was incubated for 4 hr on a rocker at room temperature, then washed out. After a final PBS wash, whole tissue was mounted onto glass slides under #1.5 cover glass and using ProLong Gold mounting media (Thermo Fisher Scientific). Digital images were taken using a Keyence BZ-X800 (Keyence Corporation, Osaka, Japan) slide scanning microscope with a 2x and 20-40x air objective. Fluorescence images were captured and stitched using the Keyence analysis software (BZ-X800 Analyzer, Keyence Corporation, Osaka, Japan). Analysis was performed using FIJI. Synapses were considered denervated if there was no NF+SV2 signal overlapping the α-BTX labeled endplate.

3.2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 and 8 (GraphPad Software) or SPSS Statistics v. 25 (IBM) software. Variables were assessed for Gaussian distribution using the D'Agostino-Pearson ($n < 50$) or Kolmogorov-Smirnov ($n \geq 50$) normality test. For normally distributed variables, statistical significance of difference was appropriately analyzed via two-

tailed t-test, one-way, or two-way, or repeated measures analysis of variance (ANOVA) with Tukey's multiple comparison post hoc analysis. All statistics are given as mean ± s.e.m. All behavioral assays and weight gain measurements included a minimum of 7 mice per group. All immunohistochemistry experiments were repeated at least 3 times. Asterisks indicate statistical significance (*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$).

3.3 Results

3.3.1 SMN upregulation by ASO administration in SMNΔ7 mice improves motor function and body weight

We have evaluated how ASO treatment affects motor function in control and SMNΔ7 mice by measuring postnatal weight gain and utilizing behavioral assays to measure righting ability and grip strength. We observed an early and profound discrepancy in postnatal weight gain between non-treated control and SMNΔ7 mice. Deficits in weight gain were detectible as early as P3 in non-treated SMNΔ7 mice (non-treated SMNΔ7 mice = 2.0029 ± 0.08443 g; non-treated WT mice = 2.4361 ± 0.10621 g; Fig. 17A-B). Upregulation of SMN by postnatal ASO administration delayed the deficit in weight gain in treated SMNΔ7 mice, with differences only becoming significant at P9 (treated SMd7 = 4.4391 ± 0.41104 g; treated WT = 5.30 ± 0.1647 g; Fig. 17A,C).

We measured grip strength (normalized by body weight) to measure motor function. We found that ASOs significantly increase the grip strength of SMNΔ7 mice after the first postnatal week (Fig. 17D). Curiously, we also found a striking but transient difference in the postnatal development of grip strength between ASO-treated and non-treated control mice (Fig. 17E). ASO-

treated WT and HET mice were significantly stronger than non-treated controls for the first few days after birth, leading to the hypothesis that ASO-mediated upregulation of SMN accelerates the neuromuscular development time course in control mice.

We next assessed the behavioral benefits of ASO administration by measuring the righting ability of SMNΔ7 mice using righting latency converted to a motor ability score (see Methods for rubric). Control mice rapidly achieved the highest score of this task assessing motor development by one week of age, while non-treated SMNΔ7 mice had severe and persistent motor impairment that showed little or no improvement over time. In contrast, ASO administration greatly improved the motor function of SMNΔ7 mice (Fig. 17F), but not to control levels.

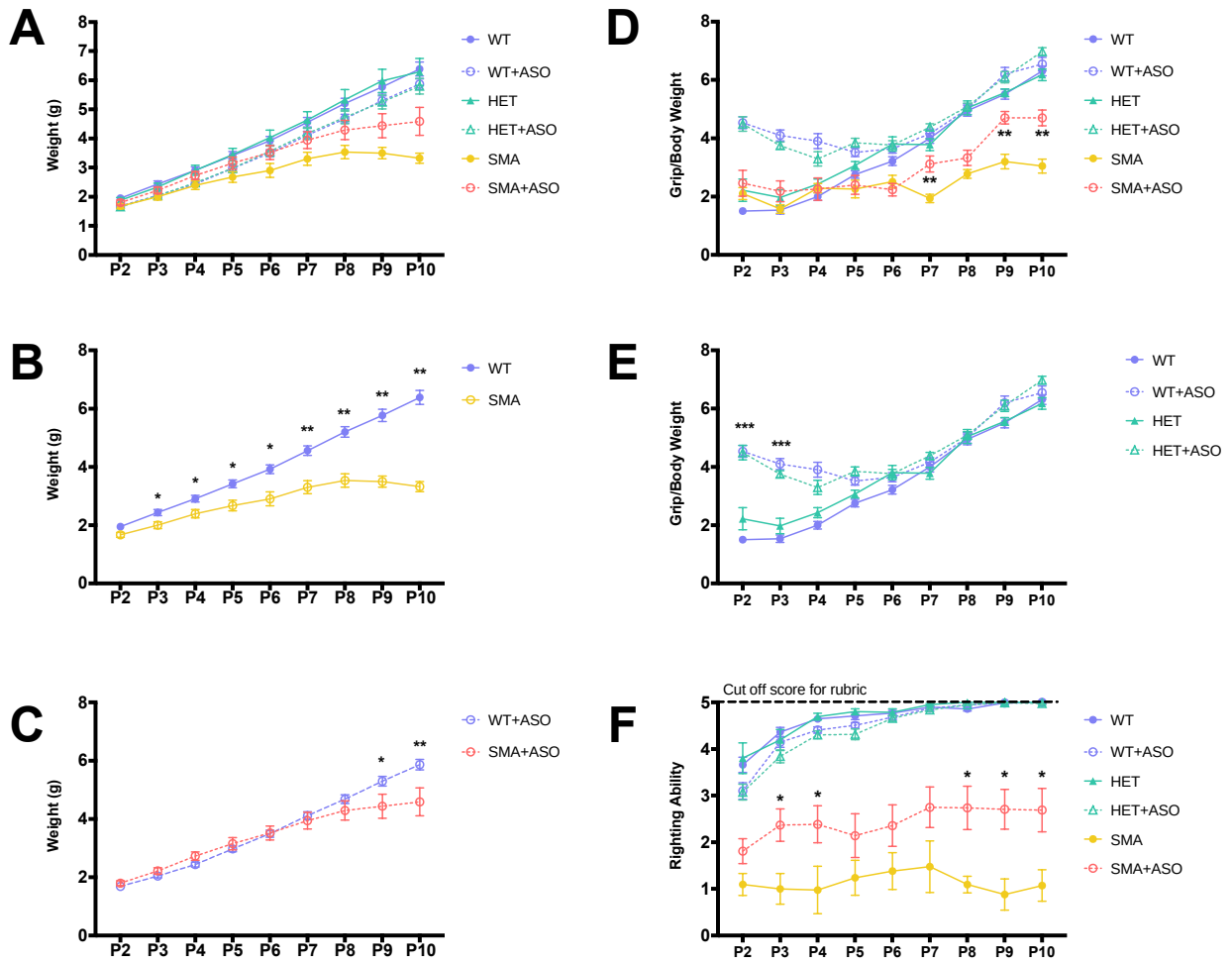

Figure 17 Improvement in weight gain and motor dysfunction following restoration of SMN by postnatal antisense oligonucleotide adinistration.

(A) Weight (in grams) for control mice (WT mice; solid purple, n = 18; HET mice; solid green, n = 10), and control mice treated with ASOs (WT+ASO, dotted purple, n = 25; HET+ASO, dotted green, n = 19), and SMNΔ7 mice (non-treated SMNΔ7, yellow, n = 8; ASO treated SMNΔ7, red, n = 11). (B) A comparison of postnatal weight gain of non-treated WT and SMNΔ7 mice from data depicted in (A), independent t-test (WT versus SMNΔ7), *p < 0.05, **p <0.005. (C) A comparison of postnatal weight gain between ASO-treated WT and SMNΔ7 mice from data depicted in (A), independent t-test (WT+ASO versus SMMd7+ASO), *p < 0.05, **p <0.005. (D) Postnatal development of grip strength (grams) of non-treated control (WT mice; solid purple), HET mice; solid green), and ASO-treated control (WT+ASO, dotted purple; HET+ASO, dotted green) mice, and SMNΔ7 mice (non-treated SMNΔ7, yellow; ASO treated SMNΔ7, red), independent t-test (SMNΔ7 versus SMNΔ7+ASO), **p < 0.005. (E) A

comparison of non-treated controls and ASO-treated controls from the data depicted in (D), ***$p < 0.0001$. (F) Righting reflex scores for WT (solid purple), WT+ASO (dotted purple), HET (solid green), HET+ASO (dotted green), SMNΔ7 (yellow) and SMNΔ7+ASO (red) mice, independent t-test of non-treated SMNΔ7 and ASO-treated SMNΔ7 mice, *$p < 0.05$.

3.3.2 Postnatal SMN upregulation improves but cannot reverse all neuromuscular pathology in the epitrochleoanconeous muscle of SMNΔ7 mice

Postnatal SMN upregulation improved but did not fully rescue differences in weight gain and motor function at the whole animal level, so we next sought to characterize changes in neuromuscular pathology. Neuromuscular denervation is an early and significant characteristic of SMA, though not all muscles are equally affected. We sought to determine the vulnerability of the ETA muscle (a muscle located in the proximal fore limb) to neuromuscular denervation by counting the percentage of endplates lacking presynaptic input. We stained whole mount epitrochleoanconeous (ETA) muscles from P11-13 mice for AChRs, neurofilament, and SV2 to quantify innervation, count endplate number per muscle, and to assess overall morphological qualities.

Qualitatively, we observed that in comparison to ETA muscles from control mice, endplates remain tightly clustered, a fraction of axon branches appeared overlapping or tangled, and overwhelming neurofilament blebbing (consuming entire endplates) was observed in distal NMJs in a large fraction of SMNΔ7 ETA muscles. ASO treatment rescued all neurofilament blebbing, though endplates remained clustered and some axon branches still appeared overlapping or tangled (Fig. 18A-D). These results suggest that postnatal upregulation of SMN is unable to reverse some of the neuromuscular pathology established during embryogenesis.

We found a main effect of genotype on the number of vacant endplates between non-treated and ASO treated mice ($F(1, 20) = 6.38$) and a trend for an interaction between ASO treatment and genotype ($F(1, 20) = 3.159$), suggesting a trend for ASOs to decrease the number of vacant endplates in ETA muscles for SMNΔ7 mice but increase the number of vacant endplates in ETA muscles of ASO-treated control mice (non-treated control mice = 0.07 ± 0.070; ASO treated control mice = 1.065 ± 0.7658; non-treated SMNΔ7 mice = 4.372 ± 1.632; treated SMNΔ7 mice = 1.814 ± 1.014; Fig. 18E). We observed modest but significant denervation (assessed as endplate vacancy, with no overlapping pre- and post-synaptic signals) in SMNΔ7 muscles, indicating the ETA muscle is mildly vulnerable to denervation.

Curiously, we observed a main effect of genotype on the number of endplates per ETA muscle (($F(1, 20) = 11.41$), $p = 0.0030$). On average, we counted ~39% more BTX spots in SMNΔ7 ETA muscles compared to non-treated controls (non-treated control BTX spots = 212 ± 23.69, n = 7; non-treated SMNΔ7 BTX spots = 293.8 ± 11.75; n = 6). We did not see a significant effect of ASO treatment on number of endplates in either genotype (ASO-treated control BTX spots = 230.5 ± 25.63, n = 6; ASO-treated SMNΔ7 BTX spots = 294.2 ± 19.35, n = 5; Fig. 18F).

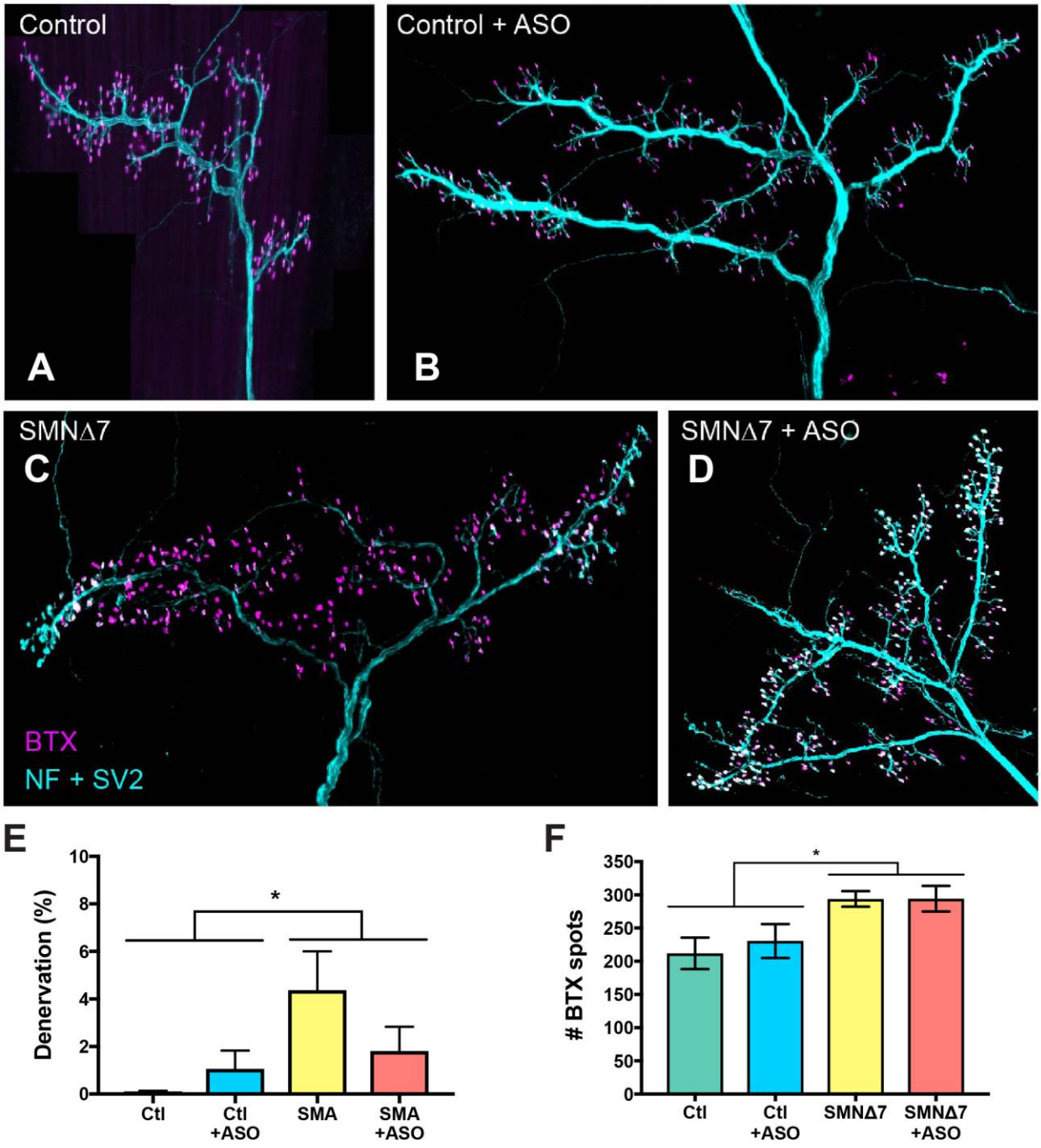

Figure 18 The epitrochleoanconeus muscle is a mildly vulnerable muscle at end stage disease in SMNΔ7 mice.

(A-D) Representative micrographs showing whole ETA muscles of P11-13 non-treated and ASO-treated control mice (E-F) Non-treated control ETA muscles, green bar, n = 7; ASO-treated control ETA muscles, blue bar, n = 6; non--treated SMNΔ7 ETA muscles, yellow bar, n = 6; ASO-treated SMNΔ7 ETA muscles, red bar, n = 5. (E)

Percentage of denervation in ETA muscles, two-way ANOVA , (genotype x ASO treatment), * $p < 0.05$. (F) The number of a-BTX labeled endplates in ETA muscles, two-way ANOVA (genotype x ASO treatment), *$p < 0.05$.

We next characterized changes in neurofilament structure and synaptic maturation using high-resolution confocal microscopy (Fig. 19). We stained whole mount ETA muscles from P11-13 mice for AChRs and neurofilament to label NMJs and innervating axons, and measured the overlapping pre- and post-synaptic signals. Accumulation of neurofilament blebbing at the nerve terminal is a defining feature of neuromuscular pathology in SMNΔ7 mice across vulnerable and resistant muscle groups, including the ETA muscle. However, ASO administration abolished terminal blebbing, suggesting that NF recycling is entirely restored despite the partial rescue of motor function. Interestingly, ASO administration did not change the density of neurofilament labeling in SMNΔ7 mouse terminals (covering ~40% of the endplate; non-treated SMNΔ7 NMJs, 39.27 ± 1.078; ASO-treated SMNΔ7 NMJs, 40.31 ± 1.718, Fig. 19E), but treatment did modify the morphological organization of neurofilament into distinct and complex branches within the nerve terminals (Fig. 19C-D). Curiously, we also found a striking effect of ASO treatment on control mice, with a significantly larger volume of neurofilament within terminals (from 22% of the NMJ as defined by postsynaptic receptor labeling in non-treated controls to 33% in ASO-treated controls (non-treated control NMJs, 22.1 ± 1.818; ASO-treated control NMJs, 33.58 ± 0.7428, Fig. 19E), suggestive of accelerated neuromuscular maturation.

We next characterized the change in presynaptic innervation of endplates. Motor endplates are innervated by more than one motor axon early in development. The timing of when motor axons reach their final targets varies according to muscle and location (i.e. around ED12.5 for the mouse diaphragm (Weichun Lin et al., 2001; Sanes & Lichtman, 1999b; Sheard & Duxson, 1997; Wu et al., 2010). Elimination of superfluous synapses occurs until only one motor input innervates

an endplate (usually by around one to two weeks of age in mice; (Sanes & Lichtman, 1999b), the rate of which also varies per muscle. The persistence of multiple motor axons innervating a single endplate at later stages of development is a hallmark feature of neuromuscular pathology in SMA mouse models. We measured the number of axons innervating each endplate and found a main effect of genotype ($F(1, 340) = 34.92$); $p < 0.0001$; Fig. 19F). On average, control mice of both treatment groups had only a single axon innervating each endplate (non-treated control NMJs = 1.01 ± 0.0139, ASO-treated control mice = 1.0 ± 0.0). In comparison, SMNΔ7 mice of both treatment groups had a significantly greater number of axons innervating each endplate (two-way ANOVA, $p < 0.0001$) non-treated SMNΔ7 mice = 1.19 ± 0.0448; ASO-treated SMNΔ7 mice = 1.29 ± 0.0464), suggesting that ASO treatment did not alter the time course of synapses elimination in the ETA muscle.

To better understand how ASO treatment affects neuromuscular development, we next assessed endplate maturation. During postnatal development, NMJs undergo a series of maturational changes to the postsynaptic acetylcholine receptor clusters in order to achieve highly efficient synaptic transmission to precisely control motor function. While it remains unclear whether motoneurons or muscle fibers regulate specific aspects of this maturation (Tintignac, Brenner, & Rüegg, 2015), it is apparent that maturation is delayed in SMA mouse models. As has previously been reported in severe SMA patients and mouse models (Harding et al., 2015; Kariya et al., 2008), we observed small and structurally simple BTX-stained endplates in ETA muscles of SMNΔ7 mice (Fig. 19G-H). In control mice, endplates were uniform in size (measured by area of BTX fluorescence) regardless of treatment (non-treated control mice = 202.3 ± 4.715 μm^2; ASO-treated control mice = 204.1 ± 4.686 μm^2). In comparison, SMNΔ7 mice had significantly smaller

endplates, regardless of treatment (non-treated SMNΔ7 mice = 144.5 ± 4.376 μm^2; ASO-treated SMNΔ7 mice = 144.3 ± 4.087 μm^2).

We also counted the number of perforations within endplates to determine if ASOs modulated endplate maturation. Similar to axonal innervation, we detected no differences between ASO-treated and non-treated controls, but we did observe a main effect of genotype ($F(1,533) = 26.15$; $p < 0.0001$). Control synapses had significantly perforations compared to SMNΔ7 synapses (non-treated control mice =0.82 ± 0.0865; ASO-treated control mice = 0.87 ± 0.0755; non-treated SMNΔ7 mice = 0.34 ± 0.0591; ASO-treated SMNΔ7 mice = 0.53 ± 0.0728; Fig. 19H).

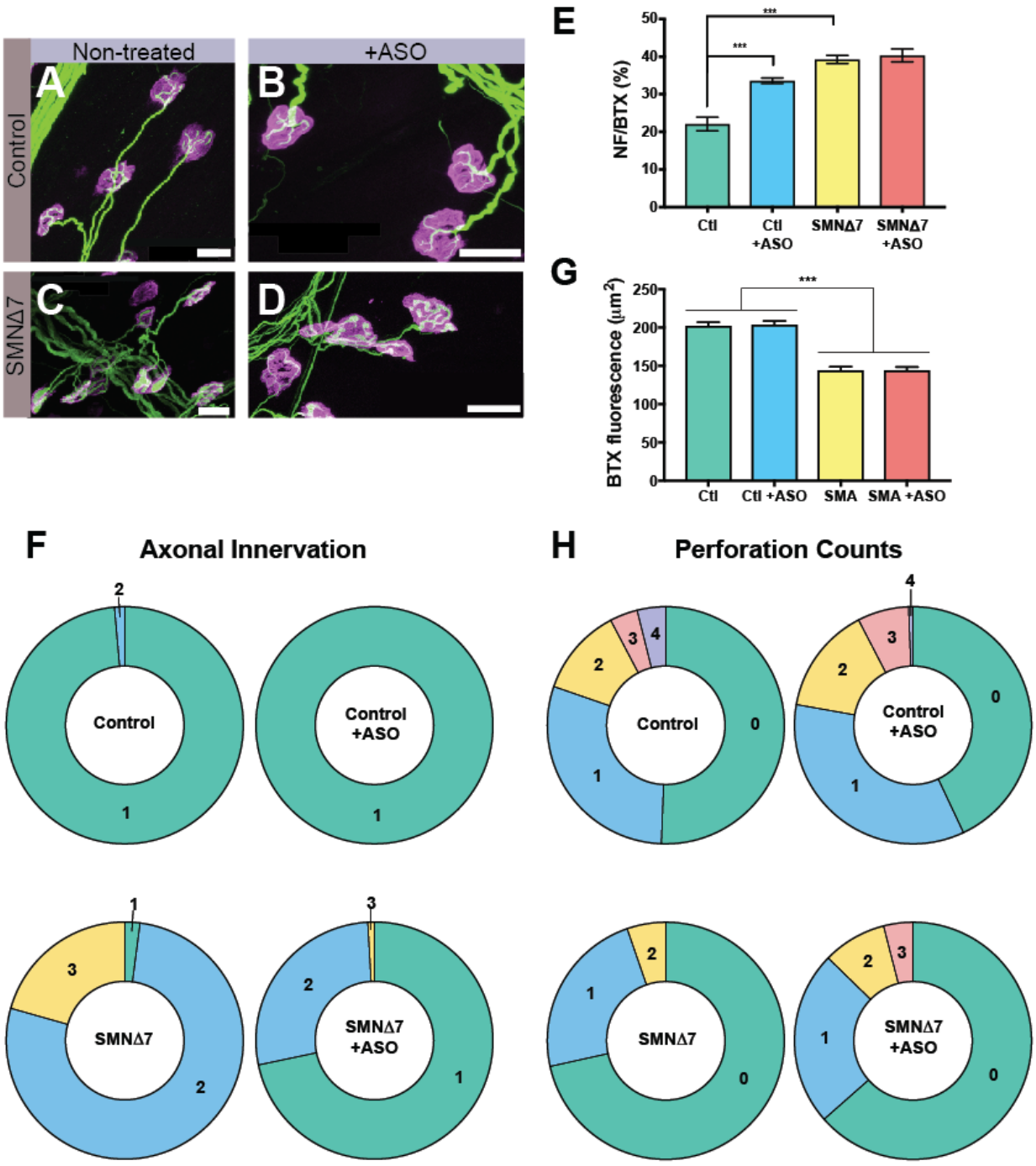

Figure 19 Delayed maturation of the epitrochleoanconeus muscle of SMNΔ7 mice.

(A-D) Representative confocal micrographs showing NMJs of non-treated and ASO-treated control and SMNΔ7 mice. The ETA muscle was labeled with α-bungarotoxin to mark acetylcholine receptors (purple) and an antibody against neurofilament (green). Scale bar represents 10 microns. (E) Quantification of anti-neurofilament labeling overlapping BTX, two-way ANOVA, ***p < 0001. (F) Donut plot showing the fraction of neurofilament

labeled axons overlapping BTX-labeled endplates with 0, 1, 2 or 3 innervating axons. (G) Quantification of BTX fluorescent area, two-way ANOVA, ***p < 0001. (H) Donut plots of the fraction of BTX-labeled endplates, with 0, 1, 2, 3, or 4 perforations.

3.3.3 GV-58 ± 3,4-DAP potentiates neurotransmission in highly vulnerable and less vulnerable muscles

Since neuromuscular development is tightly correlated with synaptic activity, we next assessed neurotransmission in the ETA muscle. It has been well established that there is dysfunctional calcium homeostasis and reduced neuromuscular transmitter release in SMA model mouse NMJs and in motoneuron cell culture models (Jablonka et al., 2007; Lyon et al., 2014; Ruiz et al., 2010; See et al., 2013; Tejero et al., 2016). Despite being less vulnerable to denervation, it is unknown whether synaptic activity was decreased in the ETA muscle from SMAΔ7 mice (Fig. 20). We measured the magnitude of transmitter release (quantified as quantal content or QC) using current clamp electrophysiology and observed a main effect of genotype ($F(1,146) = 27.38$; $p < 0.0001$). We measured a ~30% decrease in quantal content in non-treated SMNΔ7 mice compared to controls. Furthermore, we did not measure a statistically significant change in transmission in SMNΔ7 mice treated with ASOs (Fig. 20B; non-treated control quantal content = 19.89 ± 1.024; ASO-treated control quantal content = 18.96 ± 0.7729; non-treated SMNΔ7 quantal content = 14.00 ± 0.7743; ASO-treated SMNΔ7 quantal content = 15.65 ± 0.8077).

Despite the benefit of ASO treatment on neuromuscular structure and growth, persistence of pathophysiological dysfunction could explain the limited recovery of motor skills *in vivo*. Since low calcium entry and the resulting lower magnitude of synaptic transmission could contribute to motor weakness in SMNΔ7 mice, we wanted to test whether a calcium channel gating modifier

(GV-58) in combination with a voltage-gated potassium channel antagonist (3,4-DAP) would increase neuromuscular transmission in *ex vivo* NMJs (Fig. 20C-D). In control mice, application of 50 μM GV-58 increased quantal content from baseline by 50-52%, while GV-58 + 3,4-DAP doubled quantal content (non-treated control QC = 25.39 ± 1.089; ASO-treated control QC = 22.32 ± 1.431; Fig. 20B). When applied to SMNΔ7 preparations, GV-58 increased quantal content by ~80% in non-treated SMNΔ7 mice, and by 40% in ASO-treated SMNΔ7 mice. In comparison, 50 μM GV-58 + 1.5 μM 3,4-DAP further increased quantal content to 143% of baseline quantal content of non-treated SMNΔ7 mice (QC = 34.14 ± 2.774), and 80% of baseline quantal content in ASO-treated SMNΔ7 mice (QC = 28.21 ± 1.383; Fig. 20C). These results indicate that potentiation of calcium influx into nerve terminals can increase vesicle release despite the presence of structural pathology. Overall, we found that GV-58 alone was sufficient to rescue transmission to control levels in ETA muscles of non-treated and ASO-treated SMAΔ7 mouse NMJs (Fig. 20D-E), suggesting that the restoration of function in a less vulnerable muscle is achievable with an increase in calcium influx during action potential activity.

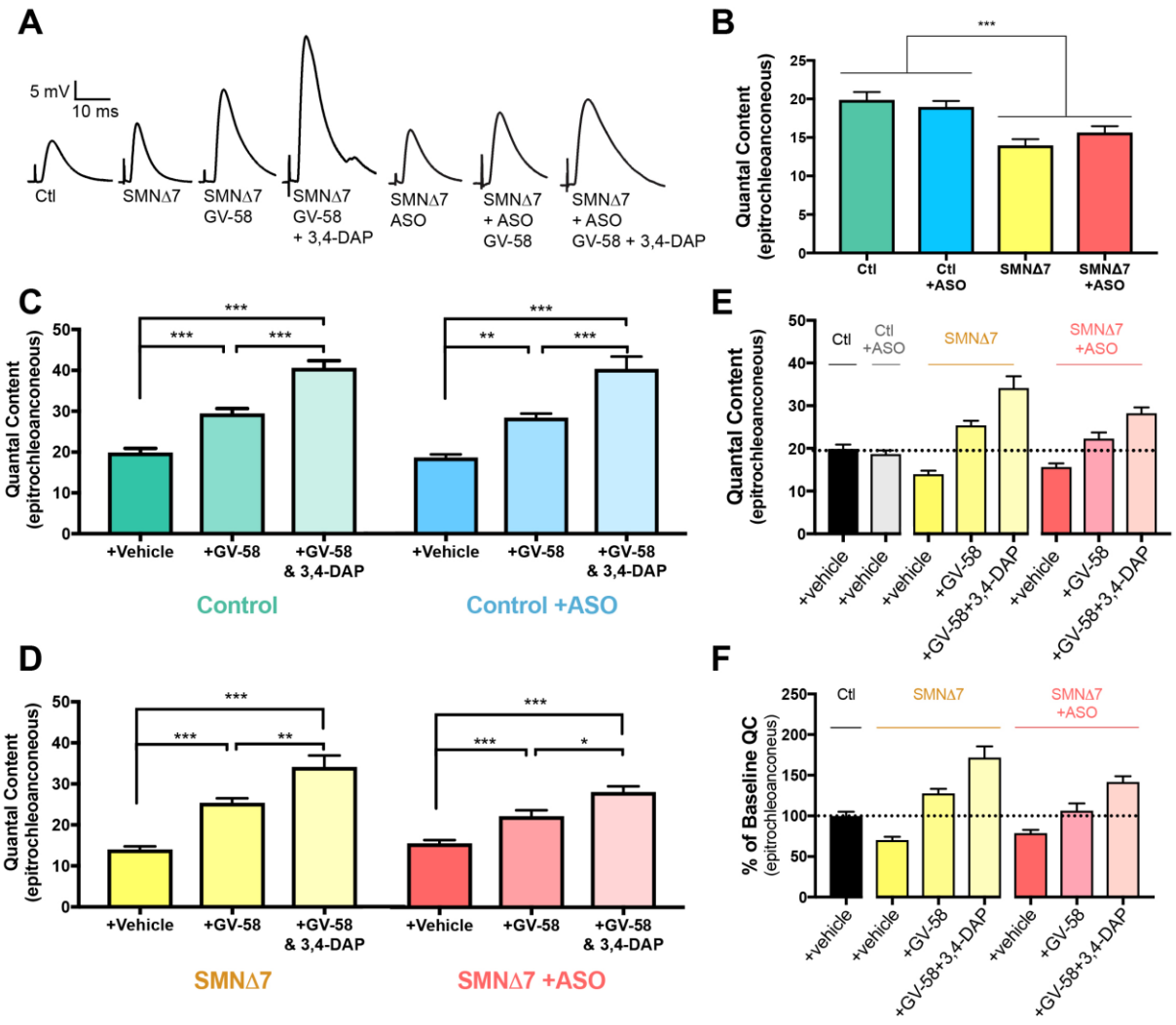

Figure 20 Persistent deficits in ETA neuromuscular transmission can be rescued by GV-58 ± 3,4-DAP.

(A) Sample traces of EPPs from control and SMNΔ7 ETA muscles. (B) Electrophysiological measurements of quantal content in the ETA muscle from P11-13 mice, non-treated controls (green bar), ASO-treated controls (blue bar), SMNΔ7 (yellow bar) and ASO-treated SMNΔ7 (red bar). Two-way ANOVA with Tukey's post hoc analysis, ***$p < 0.001$. (C-D) Bath application of 50 µM GV-58 ± 1.5 µM 3,4-DAP to the ETA muscle of non-treated control (green shades) and ASO-treated control (blue shades) mice, compared to vehicle (C), and SMNΔ7 (yellow shades), and ASO-treated SMNΔ7 (red shades) mice (D). One-way ANOVA with Tukey's post-hoc analysis, *$p < 0.05$, ** $p < 0.005$, *** = $p < 0.001$. (E) A comparison of changes in quantal content in vehicle or drug conditions. Dotted line indicates average quantal content of non-treated controls. (F) GV-58 alone is sufficient to rescue neurotransmission

deficits in epitrochleoanconeous muscles of non-treated SMNΔ7 (yellow shades) and ASO-treated SMNΔ7 mice (red shades) compared to non-treated control mice.

We next explored the effects of GV-58 ± 3,4-DAP in the transverse abdominis (TVA), which is a muscle that has previously been shown to be highly vulnerable to denervation (Murray et al., 2008), and previously established to have reduced quantal content (Ruiz et al., 2010). We found a main effect of genotype on quantal content of TVA muscles ($F(1,77) = 31.09$). Similar to what has been previously reported, we found a ~44% decrease in transmission in non-treated SMNΔ7 mice at TVA NMJs and a ~26% decrease in ASO-treated SMNΔ7 mice compared to non-treated controls (nontreated control QC = 20.49 ± 1.44; non-treated SMNΔ7 QC = 11.5 ± 0.9641; ASO-treated SMNΔ7 QC = 15.09 ± 1.929; Fig. 21B). Application of 50 μM GV-58 increased quantal content increased by 80% compared to baseline in non-treated SMNΔ7 mice (QC = 16.36 ± 1.106), and ~111% compared to baseline in ASO-treated SMNΔ7 mice (QC = 22.72 ± 1.527). Application of 50 μM GV-58 + 1.5 μM 3,4-DAP increased quantal content by ~111% compared to baseline of non-treated SMNΔ7 mice (QC = 22.83 ± 2.071), and 111% of baseline of ASO-treated SMNΔ7 mice (QC = 22.72 ± 2.821; Fig. 21C). The effect of GV-58 alone in the TVA muscle was not sufficient to restore transmission to control levels in non-treated SMNΔ7 mice (Fig. 21D-E). However, the use of GV-58 combined with 3,4-DAP was able to restore quantal content to control levels.

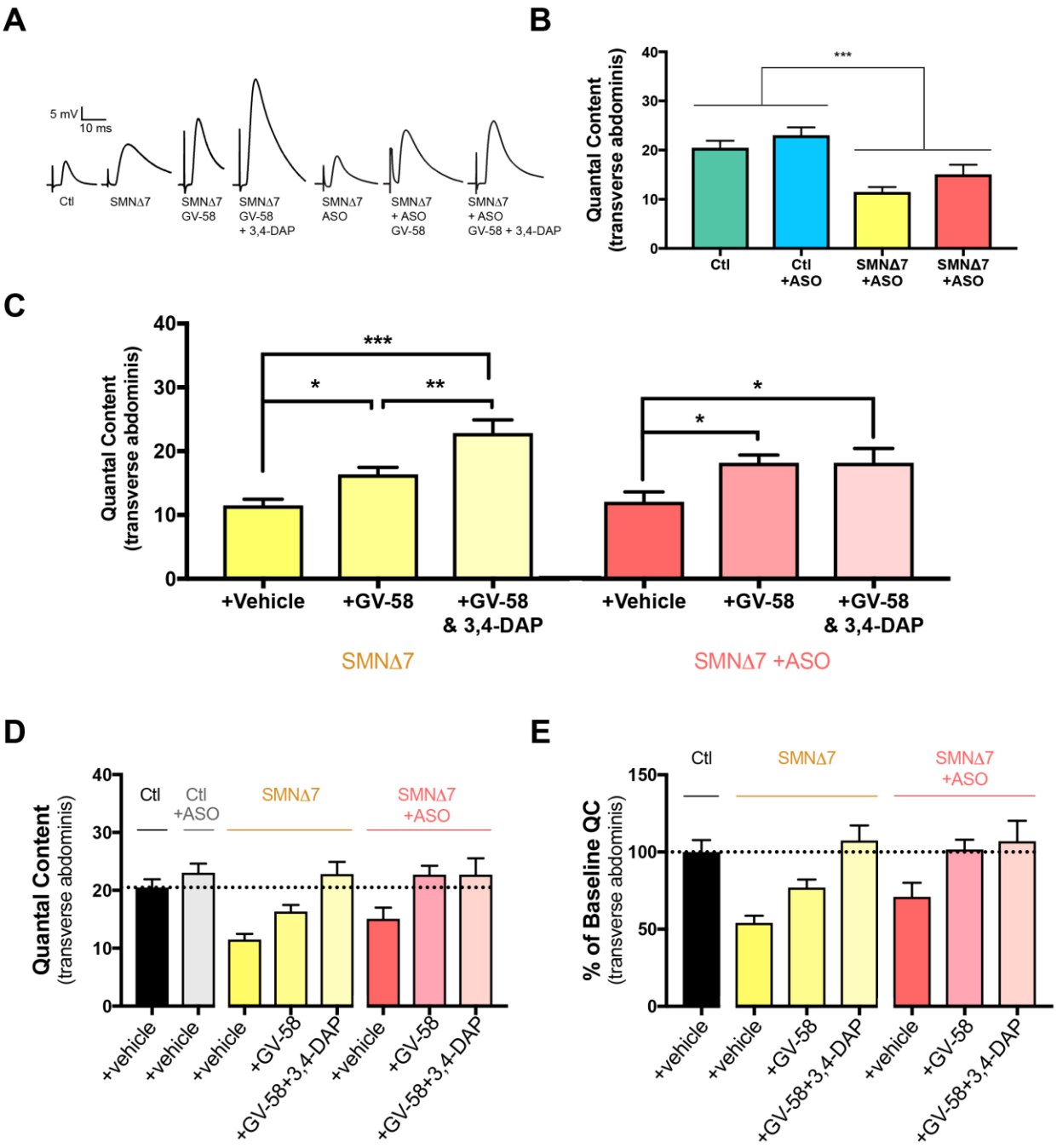

Figure 21 Persistent deficits in TVA neuromuscular transmission can be rescued by GV-58 ± 3,4-DAP.

(A) Sample traces of EPPs from control and SMNΔ7 TVA muscles. (B) Electrophysiological measurements of quantal content in the ETA muscle from P11-13 mice, non-treated controls (green bar), ASO-treated controls (blue bar), SMNΔ7 (yellow bar) and ASO-treated SMNΔ7 (red bar). Two-way ANOVA with Tukey's post hoc analysis, ***$p < 0.001$. (C) Bath application of 50 µM GV-58 ± 1.5 µM 3,4-DAP to the TVA muscle of SMNΔ7 (yellow

shades), and ASO-treated SMNΔ7 (red shades) mice compared to vehicle, one-way ANOVA with Tukey's post-hoc analysis, *p < 0.05, ** p < 0.005, *** = p < 0.001. (D) A comparison of changes in quantal content in vehicle or drug conditions. Dotted line indicates average quantal content of non-treated controls. (E) GV-58 alone is sufficient to rescue neurotransmission deficits in the TVA muscle of non-treated SMNΔ7 (yellow shades) and ASO-treated SMNΔ7 mice (red shades) compared to control mice (non-treated control, black bar, ASO-treated control, grey bar).

3.3.4 GV-58 ± 3,4-DAP increases muscle strength in SMNΔ7 mice

Despite improvements in neuromuscular development after ASO treatment, persistent deficits in neuromuscular transmission could contribute to the sustained motor impairment of ASO-treated SMNΔ7 mice (Fig. 17 & Fig. 22B). For this reason, we explored whether *in vivo* administration of GV-58 ± 3,4-DAP could benefit motor function of SMNΔ7 mice. We evaluated our treatment in P10 mice, which is near the end-stage of the disease for this mouse model. We observed an interaction of treatment and genotype ($F(1,90) = 6.754$), and main effects of genotype ($F(1,90) = 189.1$) and treatment ($F(1,90) = 25.15$; Fig. 22A). ASO-treated control and SMNΔ7 mice were significantly stronger than non-treated counterparts. To test the effect of drug administration, we measured the change in muscle strength compared to baseline 10-20 min after an acute subcutaneous administration of 150 mg/kg GV-58, alone or in combination with 0.7 mg/kg 3,4-DAP. We found no significant effects of the drug(s) in control mice of both treatment groups, comparing the change in strength after drug administration from baseline (non-treated control mice: vehicle = 106.9 ± 2.844; GV-58 = 109.8 ± 3.024; GV-58 + 3,4-DAP = 109.3 ± 1.441; ASO-treated control mice: vehicle = 109 ± 4.882; GV-58 = 114.3 ± 2.634, GV-58 + 3,4-DAP = 107.8 ± 1.426; Fig. 22B-C). In contrast, we observed that GV-58 alone was able to increase strength by ~20% in non-treated SMNΔ7 mice, and by ~34% in ASO-treated SMNΔ7 mice, compared to vehicle (non-treated SMNΔ7 mice: vehicle = 103.5 ± 3.654, GV-58 = 127.7 ± 4.149,

GV-58 + 3,4-DAP = 144.7 ± 7.494; treated SMNΔ7 mice: vehicle = 107.0 ± 3.763, GV-58 = 142.8 ± 10.36, GV-58 + 3,4-DAP = 136.6 ± 8.692; Fig. 22D-E). Interestingly, only non-treated SMNΔ7 mice significantly benefitted from the addition of 3,4-DAP to treatment, resulting in a ~39% increase in strength compared to vehicle. As ASO treatment alone improves neuromuscular maturation of SMNΔ7 mice, these data suggest that the addition of GV-58 treatment alone is sufficient to increase the synaptic transmission required to bring all the muscle fibers employed in the grip strength assay above threshold.

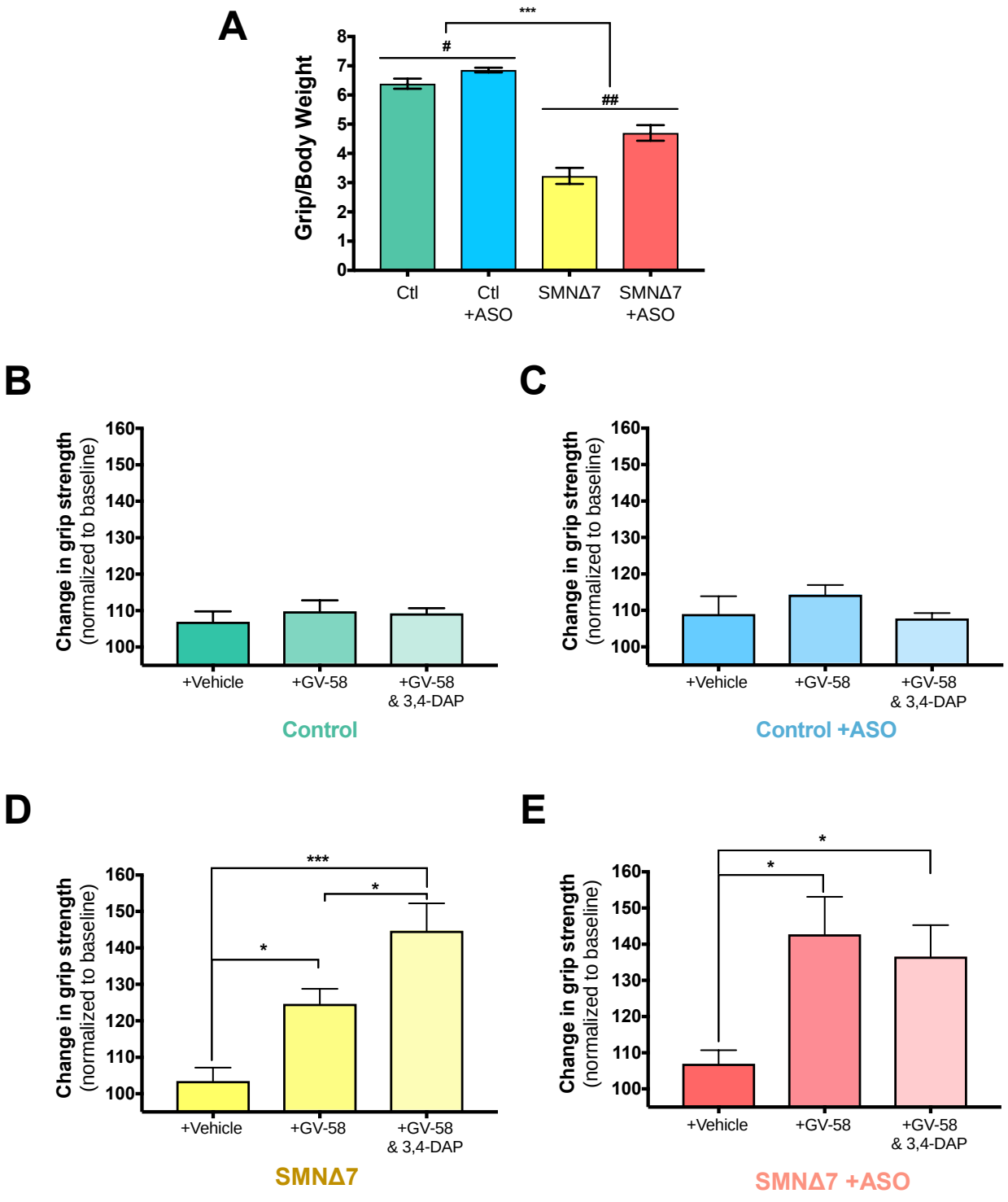

Figure 22 Improvement of strength in SMNΔ7 mice following action potential-driven augmented calcium influx into motor nerve terminals.

(A) Muscle force (in grams, normalized by weight) of P10 control (blue bar, n = 32), control + ASO (green bar, n = 46), SMNΔ7 mice (yellow bar, n = 7) and SMNΔ7 +ASO (red bar, n = 11). Two-way ANOVA with Tukey's

post hoc analysis, ***p < 0.0001, #p < 0.05, ##p < 0.001. (B-E) Acute subcutaneous injection of 150 mg/kg GV-58 ± 0.7 mg/kg 3,4-DAP in P10 pups. GV-58 ± 3,4-DAP does not significantly increase grip strength compared to the vehicle in non-treated (B) or ASO-treated (C) control mice. One-way ANOVA with Tukey's post hoc analysis, *not significant*. GV-58 administration significantly increases grip strength in non-treated (D) and ASO-treated (E) SMNΔ7 mice compared to vehicle. In non-treated SMNΔ7 mice, GV-58 + 3,4-DAP significantly improves strength compared to GV-58 alone. One-way ANOVA with Tukey's post hoc analysis, * p < 0.05, *** p < 0.001.

3.4 Discussion

Despite the immense excitement for *SMN2*-targeted ASO therapy for SMA patients, preliminary clinical observations suggest that complementary approaches will be necessary to maximize neuromuscular function (Finkel et al., 2017; Mercuri et al., 2018). We have used a mouse model to study persistent neuromuscular deficits after ASO treatment to evaluate a small molecule drug combination that can be used as a supplement to ASO therapy, or as a stand-alone treatment.

We observed significantly lower body weight as early as P3 in non-treated SMNΔ7 mice. Postnatal ASO administration improved the gain of body weight, and treated pups were not statistically different from controls until P9. In parallel, we detected improvements in motor function in SMNΔ7 mice treated with ASOs. We measured motor impairment using two behavioral assays, the righting reflex and grip strength assays. We assessed righting reflex, an assay requiring trunk, proximal and distal muscle contributions to movement, from P2-10. The poverty of righting skill in non-treated SMNΔ7 mice reflects the strenuous demand for motor function engendered by a high number of trials (six trials) and thus prolonged endurance required to repetitively achieve an upright position. In comparison, ASO-treated SMNΔ7 mice achieved about a 2 to 2.5-fold higher righting score by P10, which indicates an improvement in both motor function and

endurance. However, at no point during our evaluation did ASO-treated SMNΔ7 mice achieve a motor score similar to control mice.

We next assessed the development of postnatal grip strength from P2-10, which measures the force predominantly produced by proximal and distal limbs, with minimal utilization of trunk muscles. Non-treated SMNΔ7 mice were indiscernible from non-treated controls for about the first postnatal week. Subsequently, these SMNΔ7 mice demonstrated minimal improvement in the development of grip strength through P10. ASO treatment had genotype-dependent effects. In treated control mice, SMN upregulation via ASO treatment resulted in a dramatic increase in grip strength between P2-4. By P5, control mice were indistinguishable in strength regardless of treatment. This suggests that postnatal SMN upregulation may expedite neuromuscular growth in control mice. ASOs also modulated the development of grip strength in SMNΔ7 mice, improving strength over time but not to control levels. These data support the conclusions that trunk muscles are highly vulnerable to weakness in SMA pathology, and that proximal and distal weakness in SMNΔ7 mice is not readily detectible until about a week after birth. Furthermore, ASOs improve proximal and distal motor function, but weakness in trunk function persists in SMNΔ7 mice after ASO treatment. Finally, motor impairment was detectible in both assays prior to changes in weight in ASO-treated SMNΔ7 mice. Clinical data further support the conclusion that motor impairment may persist after ASO therapy, since only about half of the patients treated with ASOs achieve expected motor milestones (Finkel et al., 2017; Mercuri et al., 2018).

While ASO-induced postnatal SMN upregulation dramatically improved motor function and body weight of SMNΔ7 mice, we did not observe a rescue of neuromuscular transmission in muscles that are highly or minimally vulnerable near end-stage disease of these mice. We also did not observe a complete rescue of pathological NMJ morphology after ASO administration. We

grossly observed unusual axonal branching patterns in SMNΔ7 mice regardless of treatment, suggesting that established axonal aberrations are not reversible by postnatal SMN upregulation in these mice. We also did not detect a significant effect of ASO treatment on the number of innervating axons, muscle fiber endplate size, or the number of endplate perforations. Activity-dependent trophic signaling drives several of these maturational aspects. BDNF, a muscle-derived trophic signal that is regulated by neuromuscular activity, is required to stabilize neuromuscular synapses and induce pruning of superfluous axons during development (Je et al., 2013; Je et al., 2012; Lu, 2003; Yang et al., 2009). Z^+ Agrin is a nerve-derived trophic factor that is released in an activity-dependent manner and is critically involved in the topographical plaque-to-pretzel transformation of fiber endplates (Bolliger et al., 2010; McMahan, 1990). Further investigation is required to understand how transmission defects in SMA neuromuscular synapses affect trophic signaling.

We did, however, observe a significant improvement in neuromuscular denervation in SMNΔ7 mice after ASO treatment. The ETA muscle is a proximal fore limb muscle innervated by the ulnar nerve and appears to be a mildly vulnerable muscle at disease end stage. In SMNΔ7 mice, ASO treatment improved both denervation and neurofilament organization. In addition, ASO treatment of control mice increased neurofilament within nerve terminals, suggesting an acceleration of neuromuscular development. Taken together, these data suggest that some neuromuscular pathology is reversible with postnatal SMN upregulation, but some deficits established during embryogenesis persist despite treatment.

We sought to use a small molecule approach to address persistent deficits in transmission in SMNΔ7 neuromuscular synapses. It has been previously shown that (*R*)-Roscovitine, an inhibitor of cyclin-dependent kinases (cdks) with voltage-gated calcium channel agonist activity

(Meijer et al., 1997), improves neuromuscular function and neuronal differentiation in SMA cell models independently of its effects on cdks (Tejero et al., 2020). Similar to (*R*)-roscovitine, acute application of GV-58 also improved neuronal differentiation (Tejero et al., 2020). Furthermore, several other calcium-dependent modifiers have been found that dramatically rescue the SMA phenotype in some patients by improving neuromuscular function (Janzen et al., 2018; Oprea et al., 2008; Riessland et al., 2017). This evidence suggests that targeting calcium dynamics can improve the neuromuscular dysfunction caused by SMA.

In this study, we have demonstrated that a novel, first-in-class calcium channel gating modifier, GV-58, can rapidly and potently increase transmitter release from *ex vivo* SMNΔ7 motor nerve terminals, especially when combined with a voltage-gated potassium channel blocker (3,4-DAP). GV-58 ± 3,4-DAP increases neuromuscular transmission by increasing action potential-driven calcium influx into motor terminals and augmenting the release of synaptic vesicles (Tarr et al., 2014). We tested the efficacy of this novel treatment as a complement to SMN-based therapy in order to improve neuromuscular function and muscular strength.

We found that GV-58 ± 3,4-DAP effectively and rapidly increases neurotransmission in intact synapses, regardless of the severity of denervation in surrounding NMJs or the presence of motor terminal pathology (such as NF accumulation (Cifuentes-Diaz et al., 2002), reduced synaptic vesicle pools (Dale et al., 2011; Kong et al., 2009; Neve et al., 2016; Torres-Benito et al., 2011), reduced transmitter release sites (Rocío Ruiz et al., 2010a), and a paucity of calcium channel clusters (Sibylle Jablonka et al., 2007; Rocío Tejero et al., 2016) in SMNΔ7 mice). In addition to anatomical pathology, SMA motor nerve terminals have impaired calcium homeostasis (Sibylle Jablonka et al., 2007; Rocío Tejero et al., 2016) (K. See, Yadav, Giegerich, & Cheong, 2014), which may dramatically affect transmission. Altogether, these data suggest that increasing calcium

influx into motor nerve terminals is a viable strategy to augment the magnitude of transmitter release and improve neuromuscular function.

Our novel compound, GV-58, mediates its beneficial effects by directly binding to Cav2 channels (Tarr et al., 2013) and slowing the channel deactivation kinetics due to an increase in the mean open-time of the calcium channel. Because GV-58 has a greater likelihood of modifying Cav2 channels the longer they are activated, and DAP prolongs the action potential waveform duration (Ojala et al., 2020), 3,4-DAP and GV-58 synergistically enhance presynaptic calcium entry during an action potential and can dramatically reverse neuromuscular weakness caused by decreased transmission (Tarr et al., 2014). GV-58 is a synthetic derivative of (*R*)-roscovitine, and both molecules act on Cav2 channels that open during membrane depolarization (Buraei, Schofield, & Elmslie, 2007; Tarr et al., 2014; Yan et al., 2002). However, in comparison to (*R*)-Roscovitine, GV-58 has a 20-fold lower potency on Cdk inhibition (with no effect at physiological levels of ATP; (Liang et al., 2012; Tarr et al., 2012)) and a 4-fold higher efficacy for Cav2 channels, resulting in more potent Cav2 agonist effects with less off-target consequences on Cdk activity (Liang et al., 2012; Tarr et al., 2013). This difference in Cdk inhibition is critical because the activation of Cdk5 mediates the ACh-induced dispersion of AChR clusters at the developing neuromuscular junction (Fu et al., 2005; Lin et al., 2005). At the doses used in this study, GV-58 selectively targets Cav2.1 and 2.2 calcium channels (Tarr et al., 2013). Cav2.1 and Cav2.2 are the calcium channels responsible for regulating vesicle release at developing motor nerve terminals (Catterall, 2011; Urbano et al., 2008). For these reasons, GV-58 is an outstanding candidate for potentiating neuromuscular transmission with a mechanism of action specific to neurons, with limited off-target consequences.

The potassium channel blocker 3,4-DAP has been traditionally used to treat neuromuscular

weakness caused by the autoimmune disease Lambert-Eaton myasthenic syndrome (Oh et al., 2005; Titulaer, Lang, & Verschuuren, 2011; Verschuuren et al., 2006). 3,4-DAP broadens the normally brief presynaptic action potential waveform (Thomsen & Wilson, 1983) resulting in a greater percentage of open presynaptic Cav2 channels and subsequent increases in transmitter release. Therapeutic doses of 3,4-DAP achieve this broadening by blocking voltage-gated Kv3.3 channels (Ojala et al, 2020). However, dose-dependent side effects caused by 3,4-DAP infiltration of the blood-brain barrier limits the use of 3,4-DAP to achieve full symptomatic relief (Lindquist & Stangel, 2011). Interestingly, the synergistic benefit of GV-58 combined with 3,4-DAP may permit the use of a low dose of 3,4-DAP to maximize improvements to motor function while limiting side effects. This combined therapeutic approach increases calcium entry into motor terminals and potentiates evoked neurotransmitter release in both control and SMNΔ7 synapses (Fig. 22).

We have investigated the role of calcium dynamics on neuromuscular transmission in two muscles that greatly vary in their susceptibility to SMA-induced denervation. The TVA is a trunk muscle widely established to be highly vulnerable to denervation and neuromuscular pathophysiology during early disease stages (Murray et al., 2008; Ruiz et al., 2010). Due to this rapid synaptic deterioration, highly vulnerable muscles like the TVA will require an SMN-based therapeutic intervention to maximize the benefit of any neuromuscular-targeted drug that requires intact synapses to improve function. In contrast to the TVA, the ETA is a proximal muscle that is only mildly vulnerable to denervation even at end stages of the disease (Fig. 18). As such, the ETA muscle is an excellent model in which to test whether our novel therapeutic approach could increase transmission in intact but dysfunctional synapses.

Our results show an interesting discrepancy in the efficaciousness of GV-58 in rescuing

transmission deficits (Figs. 20-21). Neuromuscular transmission was restored to control levels by application of GV-58 alone in the ETA muscle, but a combination of GV-58 + 3,4-DAP was required to achieve control levels of neurotransmission in the TVA muscle. The magnitude of transmission deficits likely underlies this variable rescue.

It has been previously shown that SMA patients exhibit a progressive decrease in compound muscle action potentials (Swoboda et al., 2005), indicating that fewer muscle fibers are contributing to muscle contractions over time. Neuromuscular synapses in SMA mouse models experience reductions in neurotransmission prior to denervation (Ling et al., 2010; Park et al., 2010; Tejero et al., 2016; Kong et al., 2009; Ruiz et al., 2010). In normal conditions, the neuromuscular system accommodates dynamic physiological demands by releasing superfluous transmitter (termed 'safety factor') to reliably achieve a postsynaptic depolarization greater than the action potential threshold which subsequently causes a muscle fiber contraction (Wood & Slater, 2001). Failure to reach the action potential threshold will prevent the excitation of the postsynaptic fiber, resulting in a weaker muscle contraction. If transmission deficits in SMNΔ7 synapses result in a failure to achieve a threshold level of depolarization required for a postsynaptic action potential, then small increases in quantal content may be able to achieve this threshold. We found that GV-58 alone significantly increased the strength of disease end stage non-treated SMNΔ7 mice, and addition of 3,4-DAP further improved strength (Fig. 22). However, no difference in the change of strength after GV-58 alone or in combination with 3,4-DAP was detected in ASO-treated SMNΔ7 mice. These results indicate that ASOs partially ameliorate calcium homeostasis defects in neuromuscular terminals, and slight increases in calcium influx (induced by GV-58) is sufficient to restore motor function. Future investigations are necessary to determine whether chronic administration of GV-58 ± 3,4-DAP could mitigate or prevent the loss

of fiber participation in muscle contraction over time.

While acute administration of GV-58 ± 3,4-DAP rapidly increases the grip strength of SMNΔ7 mice, these drugs did not alter the strength of control mice. Unlike SMNΔ7 mouse synapses, control mouse synapses already satisfy the threshold for postsynaptic depolarization. Thus, increased calcium influx into control nerve terminals does lead to more transmitter release (Figs. 20-21), but this does not result in greater muscle contraction. Thus, increases in quantal content do not translate to stronger muscles in healthy conditions (Fig. 22) because these NMJs are already depolarizing muscle sufficiently for normal contraction prior to GV-58 ± DAP treatment.

We have demonstrated that targeting dysfunctional calcium homeostasis via GV-58 ± 3,4-DAP is a therapeutic approach to improving motor function. In addition, this treatment is a complementary approach to address the deficits that persist after ASO therapy (Finkel et al., 2017; Mercuri et al., 2018). Our results suggest that GV-58 may be sufficient to restore transmission deficits and improve motor function, but addition of 3,4-DAP may be of benefit for patients with delayed treatment intervention or for patients who experience suboptimal improvements in motor function. Further investigation of this second generation treatment as a complement to delayed ASO administration would elucidate these potential benefits. Additionally, chronic augmentation of synaptic activity may result in protective benefits for NMJs (Sanes & Lichtman, 1999b). Trophic factors are activity-driven molecules that support the maintenance and growth of NMJs. In addition, improvements in motor function may mitigate synaptic degradation caused by inactivity and aging (Badawi & Nishimune, 2017; Nishimune, Stanford, & Mori, 2014). Thus, increasing neuromuscular function could improve some of the indirect consequences of SMN deficiency. Though acute administration as performed in these experiments is unlikely to result in long-lasting

trophic changes or preserve neuromuscular integrity, future investigations are needed to evaluate the benefit of chronic treatment with GV-58 ± 3,4-DAP.

In contrast, an earlier intervention (before birth) with GV-58 may improve the effectiveness of ASO therapy. It has been shown that prenatal exposure to (*R*)-Roscovitine benefits the neuromuscular system and lifespan of SMNΔ7 mice by direct modulation of Cav2 channels (Tejero et al., 2020). As GV-58 is a more potent derivative of (*R*)-Roscovitine (Liang et al., 2012; Tarr et al., 2013), it is plausible that neonatal exposure to GV-58 could ameliorate some of the neuromuscular pathology and thus enhance the ability of motoneurons to benefit from ASO therapy due to decreased physiological stress postnatally at the level of the NMJ. Further research is necessary to explore this possibility.

4.0 General discussion

SMA is a leading genetic cause of death in infants, and one of the most prevalent diseases of childhood. Since the first documented cases of SMA nearly 130 years ago, the field of SMA has remarkably progressed. The 1990s saw the discovery of the *SMN1* gene and less than 30 years later, the advent of the genetically targeted therapies have dramatically altered the prognosis of patients with this rare disease.

4.1 SMN-dependent therapies: a first step in the journey to cure SMA

Prior to December 2016, no treatment existed for SMA. Patients with types 2-4 were expected to survive into adulthood, while type 0-1 forms were invariably fatal a few weeks to a few years after birth. The first disease modifying therapy to reach clinical use utilizes the intrathecally administered ASOs (nusinersen). Nusinersen has a mechanism of action on the *SMN2* gene, and corrects the splicing of this gene by interfering with an intronic splicing silencer, resulting in the increased production of full-length SMN transcripts and functional SMN protein levels within the central nervous system. Nusinersen administration dramatically improved lifespan and motor skills of infants with early or late onset of SMA beyond what would have been possible during the natural disease progression (Finkel et al., 2017; Mercuri et al., 2018). Since then, nusinersen has been approved by the European Medicines Agency in 2017, though individual European country adoptions of this treatment have been gradual.

4.1.1 Limitations of SMN-dependent therapy

Over the last three years, many SMA patients worldwide have been treated with nusinersen. These patients comprise a broad range of SMA types, age of symptom onset, and extent of symptoms at therapeutic intervention. Information about tolerability and efficacy of nusinersen treatment in different clinical settings and age groups is now emerging (Darras et al., 2019). Overall, ASOs appear to positively modulate disease severity in patients with SMA types 1-3. However, the risk of potentially serious adverse side effects has resulted in debate about treating presymptomatic infants with four or more copies of *SMN2* (Darras & Vivo, 2018; Glascock et al., 2012).

Another SMA population that has been questioned for nusinersen eligibility is adults that have already experienced significant motoneuron loss. Data from infants and children indicate that the best therapeutic benefits were seen in patients receiving early diagnosis and treatment (Darras & Vivo, 2018; Finkel et al., 2017; Mercuri et al., 2018). The clear benefit of early treatment is supported by autopsy evidence showing the highest demand for SMN expression in the CNS and skeletal muscle during the perinatal period. However, despite the decrease in expression after birth, SMN is still required throughout life and differences in SMN levels between patients and the healthy population are apparent at all ages (Ramos et al., 2019). Thus, an opportunity for SMN-based therapeutic intervention still exists in adults (Wadman et al., 2017). Evaluation of eligibility for nusinersen demands a consideration of whether the high cost, potentially serious risk events associated with repetitive and invasive administration, and possibly limited improvements after therapy would be ultimately beneficial for adults experiencing advanced disease progression. One study evaluating compound muscle action potentials in adult SMA patients that have undergone 14 months of nusinersen treatment found persistent neuromuscular failure, regardless of the

ambulation status of the patient (Elsheikh, 2020). One possible reason for the minimal improvements seen in preliminary investigations of nusinersen treatment of adults with advanced stages of SMA (Wadman et al., 2017) is the uniform dose of nusinersen (12mg per six months), regardless of age, body weight, or disease severity. Another reason may be related to central penetrability, as a rostral to caudal gradient of nusinersen distribution has been observed in autopsy spinal cords of severely affected patients (Ramos et al., 2019), as well as peripheral penetrability, which is restricted in patients with intact blood-brain barriers.

In May 2019, a second SMN-based therapy was approved by the United States Federal Drug Administration. Zolgensma is an intravenously administered adeno-associated virus serotype 9 that inserts a copy of *SMN1* into motoneurons and other central and peripheral cells (Mendell et al., 2017). This single-use therapy is limited to children under 2 years of age to ensure blood-brain penetrability of the systemically administered AAV9. This AAV9 therapy may be more beneficial than ASOs for patients receiving early intervention. ASOs correct *SMN2* splicing, but likely do not produce as much full-length SMN as insertion of the *SMN1* gene, unless a patient expresses high copy numbers of *SMN2*. Small cohorts have been treated with the AAV9 for a relatively short amount of time, limiting our understanding of the long-term outcomes of this therapy. Therefore, more historical data are necessary to be able to compare the tolerability and effectiveness of AAV9 and ASO therapies. While a few children who have received Zolgensma therapy have switched to nusinersen, the reason for the switch and the outcome of the change are unknown. No reports have been published to date on concurrent or sequential use of Zolgensma and nusinersen (Pearson et al., 2019).

4.1.2 SMN-dependent therapies challenge traditional classification notions

Thus far, genetic approaches to treat SMA have dramatically altered the traditional classification of patients. Historically, patients were grouped into five nominal classification types (0-4) depending on age of onset and achieved motor milestones, and were helpful to predict motor function and disease progression. However, improvements in therapeutic options, nutrition, and ventilation as well as genetically targeted therapies have dynamically altered the disease history and an overlap in clinical characteristics among SMA types are common today. Additionally, patients receiving SMN-dependent therapy are likely to transcend single categories over time, though more historical data are necessary to understand treatment outcomes. Thus, the usefulness of this type of clinical classification is diminished. Alternatively, a classification system describing current motor function and thus therapy response has been suggested (Wirth et al., 2020), categorizing patients as "nonsitters", "sitters", and "walkers" (Finkel et al., 2018; Mercuri et al., 2018). This type of classification acknowledges that the SMA phenotype is a continuum rather than limited to a restricted prediction.

4.2 Use of biomarkers to assess response to SMA therapy

Biomarkers in SMA are characteristics that can be objectively measured and evaluated to assess the pathological processes and biological response(s) to a therapeutic intervention. These markers can be used to predict disease severity, prognosis and therapeutic efficacy. The last few years have seen an increase in the research for biomarkers in SMA, and include molecular (Kolb et al., 2006; Tiziano et al., 2012), physiological (Günther et al., 2019; Querin et al., 2018),

structural (Bonati et al., 2017), and clinical markers (Krosschell et al., 2018). However, the most promising results have come from blood-derived molecular biomarkers, as these results are not confounded by assessor bias, inter-rater variability, and dependency on extensive patient cooperation. SMA biomarkers derived from blood samples are quantitative, unbiased and require minimal invasiveness (Navarrete-Opazo, Garrison, & Waite, 2020).

SMN-related biomarkers have been extensively investigated, including *SMN2* copy number, and SMN transcript and protein levels (Crawford et al., 2012; Czech et al., 2015; Vezain et al., 2007). Generally, these markers best predict disease severity in type 1 patients, but not type 3. While *SMN2* copy number is generally correlated with disease severity, positive or negative modifiers of the SMA phenotype restrict the sole use of *SMN2* to determine disease severity (Crawford et al., 2012; Janzen et al., 2018; Oprea et al., 2008; Riessland et al., 2017; Vezain et al., 2007). Assessment of *SMN2* mRNA transcripts and Hammersmith Functional Motor Scale (a common motor assay used to evaluate the motor function of SMA patients) were not necessarily correlated (Tiziano et al., 2012; Vezain et al., 2007). Similarly, full-length or truncated SMN was not found to correlate with disease phenotype (Crawford et al., 2012; Czech et al., 2015; Kolb et al., 2016; Sumner et al., 2006; Wadman et al., 2016). Several of these investigations utilized endogenous housekeeping genes, which may vary widely across the general population (Bustin, 2000), and may confound results if affected by the SMN-based treatment. Use of SMN-based biomarkers for individual assessment may benefit response to therapeutic intervention, but have not been reliably proven to predict disease severity or motor function.

The most promising biomarker for both disease severity and response to therapeutic intervention in SMA is the plasma phosphorylated neurofilament heavy chain (pNF-H). Neurofilament biomarkers have been investigated in multiple neurological diseases, such as ALS

(C.-H. Lu et al., 2012), multiple sclerosis (Lycke et al., 1998), Parkinson's, and Alzheimer's disease (Lin et al., 2018), as well as injury induced by preeclampsia (Evers et al., 2018) or traumatic brain injury (Shahim et al., 2016). Evaluation of pNF-H in type 1 patients enrolled in the nusinersen ENDEAR clinical trial (Darras, 2019) showed 10x greater plasma levels compared to healthy controls. These levels were inversely correlated with multiple markers known to impact disease severity, including time of first dose, age of SMA diagnosis and symptom onset, and Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND; another common assay to evaluate motor function in SMA patients). Levels of pNF-H declined as a function of aging in both treated patients and healthy controls, which may reflect normal neuronal pruning in the central nervous system, but pNF-H levels declined to a greater extent in the treated patients (Darras et al., 2019). This additional decrease may be due to the corrected recycling of neurofilament accumulations that appear within motor nerve terminals prior to treatment.

While pNF-H is a promising candidate biomarker to assess effectiveness of SMN-based therapy, a complementary treatment targeting neuromuscular function may not be appropriately evaluated using pNF-H levels. Mono-innervation of neuromuscular terminals in humans is achieved prior to birth (MacIntosh et al., 2006), so even delays in axonal pruning at neuromuscular synapses may not extend long past the perinatal period. If chronic increases in neuromuscular transmission accelerate the elimination of lingering neuromuscular poly-innervation (thus increasing pNF-H in plasma due to the shedding of deteriorating superfluous NMJ terminals), pNF-H levels are unlikely to be detectable for an extended duration. Thus, a different biomarker may provide better information on the efficacy of chronic neurotransmitter augmentation. One potential molecular biomarker is mature BDNF, which is released and cleaved in an activity-dependent manner from NMJs. This neurotrophin has been previously assessed as a biomarker for

the therapeutic readout on the effectiveness of repetitive transcranial magnetic stimulation for schizophrenia and depression (Peng et al., 2018), as well as a diagnostic marker of bipolar disorder (Fernandes et al., 2015), mesothelioma cancer (Smeele et al., 2018), and dynapenia (the age-related decline of NMJs and skeletal muscles) (Kalinkovich & Livshits, 2015). Another neurotrophin that may be an effective biomarker is IGF-1. This neurotrophin has been previously shown to increase in expression after exercise in intermediate SMA mice and correlate with improved motor function (Biondi et al., 2008). IGF-1 has been previously explored as a biomarker for melanoma cancer (Frenkel et al., 2013), Parkinson's disease (Sherbaf et al., 2018), frailty induced by aging (Cardoso et al., 2018), as well as to measure response to growth hormone treatment (Bielohuby et al., 2014).

The best biomarker candidate to assess improvements in neuromuscular function, however, is not molecular. Compound muscle action potentials (CMAP) have already been demonstrated to correlate with motor function and disease severity (Kolb et al., 2016; Swoboda et al., 2005), providing a discernible baseline of function. Reports on the change of CMAP amplitude after nusinersen treatment indicate ASOs significantly improve but do not completely prevent neuromuscular failure (Bishop, Montes, & Finkel, 2018; Finkel et al., 2017; Paton, 2017). Thus, CMAP amplitude can be used as an electrophysiological biomarker to evaluate neuromuscular improvement after therapeutic intervention(s). Furthermore, this type of biomarker can be rapidly and repetitively assessed with minimal invasiveness, permitting the evaluation of fast-acting SMN-independent drugs, or monitoring neuromuscular function over time.

While not an objective biomarker of therapeutic response, it is also crucial to assess the treatment impact on emotional and mental wellbeing, as well as any changes to quality of life experienced by patients and/or their caregivers. SMA may be a multi-organ disease comprised of detectible pathology across organ systems, but patients (especially older ones) experience other

disease-associated stressors that are not easily observable using molecular or electrophysiological measurements. One's perception of wellbeing is a critical component of subjective happiness and satisfaction with life (Diener et al., 1999). Thus, biological benefits should be assessed alongside psychological benefits, particularly for patients who are old enough to have personal goals (such as improved strength or motor skills), endure exceptional psychological stress (such as making or enduring difficult medical decisions or contemplating the plateau or loss of motor function), and encompass self-identity (and the consequences of dependence on caregivers). A true "cure" for SMA will not just dramatically improve biological dysfunction but boost psychological health, as well.

4.3 Future directions of SMA therapy

The long-term success of SMA therapy depends on the extent of improvement and permanency of recovery. For most patients, the best therapy to salvage motor function and lifespan will be SMN-dependent. This conclusion is supported by compelling and vast evidence from mouse models and patient tissue demonstrating that SMN is crucial for motoneurons. SMN-dependent therapy is also necessary for the development and maintenance of a variety of peripheral organs, including skeletal muscle, heart, kidney, liver, pancreas, spleen, vasculature, bone and connective tissue, gastrointestinal tract and the autonomic nervous system (Yeo & Darras, 2020). Currently FDA approved therapies for SMA (nusinersen and Zolgensma) do not fully rescue motor impairment or development for all patients, particularly those who have few *SMN2* copy numbers and/or receive delayed treatment. The future of SMA therapy for patients who do not receive full

symptomatic relief from SMN-dependent therapy will require additional supportive treatments. Additionally, patients who are either ineligible for, or do not receive, SMN-based treatment (due to cost, availability, access, or condition) will require SMN-independent strategies to improve motor function. Our work demonstrates the benefit of a neuromuscular-targeted, SMN-independent approach to treat residual motor dysfunction with or without ASO therapy. We have demonstrated the effectiveness of augmenting neuromuscular transmission to improve strength in SMNΔ7 mice.

4.3.1 Finding a cure through complementary treatment

Muscle strength and endurance (and consequently motor skill) are driven by neuromuscular activity. Motor skills permit the performance of activities required for daily living, including movement between rooms or to/from a bed, shower, or toilet, as well as for meal preparation and eating, hygienic practices, and (in the modern age) use of a keyboard and mouse. The ability to perform these activities would provide meaningful clinical improvement to patients and their caregivers (Hjorth et al., 2017; McGraw et al., 2017). SMN-based therapy is a remarkable start to improving motor function, but patients that respond sub-optimally to treatment would benefit from complementary, SMN-independent medicine to further improve motor skills. Our results in SMNΔ7 mice and other investigations of patients receiving nusinersen (Bishop et al., 2018; Finkel, et al., 2017; Paton, 2017) demonstrate that neuromuscular weakness persists after ASO treatment. The discovery of SMA phenotypic modifiers further supports the notion that the protection of NMJs can significantly improve disease progression. PLS3, CHP1, and NCALD are SMN-independent modifiers that are all associated with restoration or protection of neuromuscular integrity and function (Janzen et al., 2018; Oprea et al., 2008; Riessland et al., 2017). Though the

exact mechanisms through which these modifiers exhibit protection is unclear, it does suggest that NMJ stability is a crucial component of SMA pathogenesis. Yet there remains a gap in SMA therapies that directly protect neuromuscular function, despite the potential to drastically improve patient fatigue, independence, and quality of life (McGraw et al., 2017).

4.3.2 The need for therapeutic strategies to target neuromuscular function

High levels of SMN are primarily restricted to the timeframe of neuromuscular development, much of which occurs during embryogenesis (MacIntosh et al., 2006). While it is clear that postnatal SMN upregulation has remarkable benefits to patients regardless of severity or symptom onset prior to treatment, neuromuscular deficits persist after treatment. Historical evidence of nusinersen therapy has demonstrated that not all patients achieve normal motor development (Finkel et al., 2017; Mercuri et al., 2018). Furthermore, several reports have demonstrated that NMJs remain vulnerable to dysfunction after nusinersen treatment, as observed through measured reductions in CMAP amplitudes (Bishop et al., 2018; Paton, 2017). Two reasons may underlie this neuromuscular failure. Firstly, it is possible that some motoneurons remain vulnerable to neuromuscular denervation, but collateral sprouting prevents muscle atrophy. Secondly, prior to denervation, NMJs may experience deficits in neuromuscular transmission that may preclude postsynaptic excitation and contraction. Our results showed no effect of ASOs on mitigating the deficits in quantal content in highly and mildly vulnerable muscles of the SMNΔ7 mouse. It is possible that local SMN roles at NMJs are unsatisfied by central SMN upregulation, or perhaps postnatal SMN upregulation is insufficient to counteract established pathology.

It should be noted that there is a striking difference between the quantal content of NMJs between humans and mice. On average, human quantal content ranges from 20-25 (with a safety

factor of about 2), compared to the quantal content of 50-75 in mice (with a safety factor of bout 4) (Slater, 2008). If the reduction in quantal content widely observed in SMA mouse NMJs is also true in patient NMJs, then even slight decreases in transmission would have magnified effects on muscular contraction. In contrast, slight increases in quantal content, such as those achieved by low doses of GV-58 ± 3,4-DAP, may rescue postsynaptic excitation. Denervation and/or reduced transmission could cause the electrophysiological defects (measured as reduced CMAP amplitudes) present in patients after treatment. After all, if neurotransmission deficits persistent throughout development, activity-driven neurotrophic factors may be insufficient to support synaptic maintenance and could consequently drive axonal retraction.

4.3.2.1 If not then, why now? Why we expect GV-58 ± 3,4-DAP is a better neuromuscular medicine for SMA

Use of neuromuscular-targeted drugs for SMA is a natural notion to improve the motor function of patients. For decades, patients with neuromuscular disease have been treated with a variety of targeted drugs to improve synaptic communication. Myasthenia gravis patients often benefit from cholinesterase inhibitors such as pyridostigmine, which delay the degradation of acetylcholine in the synaptic cleft. Preliminary studies investigating pyridostigmine found some benefit to SMA patients (der Pol et al., 2012). Similarly, aminopyridines are currently being evaluated for SMA type 2-3 patients (Wadman et al., 2020). Aminopyridines, which have been used for several decades to treat Lambert-Eaton myasthenia syndrome and multiple sclerosis, provide partial relief from weakness. However, widespread use of these neuromuscular-targeted drugs is not seen for SMA patients. Should we expect that the clinical use of GV-58 would provide exceptional benefits in comparison to these other NMJ-specific drugs? The answer depends on several factors. Firstly, any patient with a severe or intermediate SMA phenotype would maximally

benefit from any neuromuscular-targeted drug when used as a complement to SMN-based therapy. Similar to the treatments used for the autoimmune diseases listed above, GV-58 ± 3,4-DAP does not address the cause of this motoneuron disease and thus does not treat the other motoneuron or peripheral symptoms caused by deficient SMN levels. Yet NMJ function is still a critical therapeutic target in SMA, illuminating the complexity of treatment required to "cure" this disease. In support of this conclusion, evidence from Courtney et al (2019) demonstrated that blocking the p53 (death-associated) pathway in SMA mice can prevent neuromuscular denervation but does not halt motoneuron degeneration (Courtney et al., 2019). Yet prevention of motoneuron death via SMN-based therapy does not preclude neuromuscular failure in nusinersen-treated patients (Bishop et al., 2018; Paton, 2017). This evidence suggests that SMA pathology in motoneurons has multiple loci that require targeted, complementary treatments to maximize function.

A second factor influencing the potential efficacy of GV-58 is the timing of intervention. Tejero et al (2020) demonstrated that administration of the GV-58 parent molecule, (*R*)-Roscovitine, to pregnant dams dramatically improved the lifespan and function of SMA mice via its action on voltage-gated calcium channels (Tejero et al., 2020). SMA is partially a disease of neuromuscular maturation, which begins early in gestation in humans (Martínez-Hernández et al., 2013) and is substantially completed by birth (MacIntosh et al., 2006). If early administration of GV-58 ± 3,4-DAP (in particular, prenatally) can increase calcium influx into nerve terminals during the critical, activity-dependent window of neuromuscular development, then it is possible that GV-58 can have outstanding effects, particularly when used as a combination to potentiate calcium influx. Furthermore, if neuromuscular dysfunction is, at least in part, due to disrupted calcium homeostasis, then GV-58 + 3,4-DAP would specifically address the underlying cause of pathology. In fact, this hypothesis is supported by our data and previously published work. Tejero

and colleagues (2016) have shown that neuromuscular transmission is indiscernible between control and SMNΔ7 mice at low (1 mM) calcium, but transmitter defects arise at physiological (2 mM) calcium (Tejero et al., 2016). Results from our *in vivo* drug treatment further support the hypothesis of calcium deficiency. The combination of GV-58 + 3,4-DAP was more effective *in vivo* than GV-58 alone only in non-treated SMNΔ7 mice, suggesting that calcium influx defects were greater than those of ASO-treated mice. This is not surprising, as Doktor and colleagues (2016) observed a decrease in the splicing of the pore-forming unit of voltage gated calcium channels in P1 SMNΔ7 mice that were restored with ASO treatment (Doktor et al., 2017). Thus, if insufficient calcium influx is responsible for several aspects of neuromuscular pathology, then one could expect a robust clinical response to GV-58 + 3,4-DAP, especially for patients expressing few *SMN2* copy numbers or those receiving early intervention.

A final factor to consider is the intrinsic variability of patients that is poorly understood. SMA is a complex disease with underlying factors influencing overall phenotype, including number of *SMN2* copies and the presence (or absence) of protective modifiers. This notion of variability is illuminated by the existence of discordant families (Oprea et al., 2008) as well as by patients that express protective modifiers but do not experience protective effects (Hasanzad et al., 2010; McGovern et al., 2015). Thus, some patients may benefit more from GV-58 than others, which would be in line with what has been reported in preliminary trials of pyridostigmine (der Pol et al., 2012). However, any treatment that provides clinically meaningful benefits to a subset of patients is worthy of investigation.

4.3.2.2 Temporal optimization of NMJ-targeted interventions

With the increase in awareness of SMA due to the advent of disease-modifying therapies, presymptomatic diagnosis of SMA is now more common (Khaniani, Derakhshan, & Abasalizadeh,

2013). Parents that are aware of being carriers of heterozygous *SMN1* deletion/mutation (due to carrier screening or because one or more of their children is affected by SMA) may elect to prenatally test the fetus for the presence of functional *SMN1*. Prenatal genetic studies of *SMN1* can occur as early as 11-14 weeks via chorionic villus sampling or after 14 weeks via analysis of amniotic fluid. More recent advancements in haplotype testing allows for non-invasive prenatal testing using maternal blood (Parks et al., 2017). Earlier detection of SMA allows for the treatment of newborns presymptomatically, but no treatment has been approved for use prior to birth despite the appearance of perinatal symptoms (Macleod et al., 1999).

When would be the optimal time for intervention for prenatally diagnosed patients? A comparative analysis of healthy and genetic-positive fetuses (null *SMN1* and 1-2 copies of *SMN2*) between 11-14 weeks did not find differences in movement, indicating neuromuscular dysfunction begins after this gestational age. However, diminished movements have been reported during the third trimester of gestation in patients with severe SMA (Macleod et al., 1999). Studies of predicted SMA type 1 fetal myotubes (1-2 copies of *SMN2*) at 12-15 weeks of age are smaller than controls (Martínez-Hernández et al., 2009). Furthermore, Martinez-Hernandez and colleagues (2013) observed both clustered and dispersed acetylcholine receptors at 12 weeks, but major fragmentation of postsynaptic receptors at 14 weeks coinciding with innervation by multiple axons (some with irregular branching patterns). Curiously, human NMJs of predicted type 2 SMA (3 copies of *SMN2*) did not differ from controls between 12-14 weeks (Martínez-Hernández et al., 2013). This evidence suggests that endplate destabilization occurs after neuromuscular innervation (Martínez-Hernández et al., 2013).

Other ultrastructural NMJ defects are apparent during neuromuscular development, including densities of synaptic vesicles far from release sites in human tissue (Martínez-Hernández

et al., 2013), and a paucity of release sites and voltage-gated calcium channels in mouse models (Dachs et al., 2011; Jablonka et al., 2007; Tejero et al., 2016). These ultrastructural defects precede changes in motor activity, suggestive of a presymptomatic temporal target for patients predicted to develop severe forms of SMA. While prenatal diagnosis permits the monitoring of symptom development *in utero*, not enough is understood about neuromuscular pathology during embryonic stages in humans to determine an optimal time for therapeutic intervention of all SMA types. Yet mouse models have clearly illuminated the critical impact of early intervention, both for SMN-dependent and independent therapies (Bowerman et al., 2012; Naryshkin et al., 2014; Paez-Colasante et al., 2013; Tejero et al., 2020).

Could reduced neuromuscular transmission underlie denervation in SMA? If so, when does deficient NMJ transmission begin, and are deficits modulated by phenotype severity? Decreased quantal content has been observed in mild and severe SMA mice (Ruiz et al., 2010; Ruiz & Tabares, 2014), suggesting that reduced transmission is a prominent symptom of any deleterious decrease in SMN. However, mildly reduced SMN permits homeostatic compensation for synaptic denervation, while severely reduced SMN may incur transmission deficits too early for the motor system to be able to compensate. Alternatively, transmission deficits may only be a symptom of low calcium influx into terminals, and other calcium-dependent mechanisms could regulate synaptic dissolution.

Could delivery of GV-58 during gestation improve the predicted SMA phenotype? Daily systemic delivery of (*R*)-Roscovitine to pregnant dams was sufficient to significantly improve neuromuscular pathology and lifespan of SMA mice (Tejero et al., 2020), suggesting that once-daily oral delivery of GV-58 to pregnant mothers could improve the prognosis of SMA babies. Safety and toxicity of prenatal administration will require extensive evaluation, but the potentially

dramatic benefit to patients is worthy of investigation. Additionally, other factors must be considered, including the timing and duration of administration. Understanding the optimal temporal intervention requires consideration of the impact of disease modifiers and the loose correlation of *SMN2* copy number and predicted outcome. Transgenic and inducible mouse models of SMA will be an excellent platform to explore these questions. Clearly, the best treatment would be one that can be administered prior to or at the onset of early neuromuscular symptoms, but the field needs better understanding of when neuromuscular pathology begins across the continuum of SMA phenotypes to optimize and personalize a treatment strategy.

Finally, for the many patients who currently experience debilitating or restrictive motor symptoms of SMA, the only optimal time for neuromuscular-targeted therapeutic intervention is "NOW". Results from our study of acute GV-58 administration in non-treated SMNΔ7 mice (an intermediate phenotype) and ASO-treated SMNΔ7 mice (a more mild phenotype) show that rapid improvement in strength is achievable well after neuromuscular defects have been established. A thorough investigation using mild SMA model mice would further elucidate the potential benefits of GV-58 administration in the presence of minor motor dysfunction but the absence of SMN upregulation.

4.4 Potential neurotrophic effects caused by chronic potentiation of neuromuscular transmission

Our results have demonstrated that acute potentiation of NMJ activity can result in rapid increases in transmission and muscle contraction, and it is known that synaptic activity plays a crucial role in neuromuscular development and maturation. However, the acute use of GV-58 ±

3,4-DAP is unlikely to have long-lasting protective effects on NMJ integrity. How would chronic use of GV-58 ± 3,4-DAP affect NMJ stability over time? Further investigation is necessary to elucidate these potential benefits, but one conjecture is that long-term potentiation of neuromuscular transmission results in an increase in trophic signaling.

Neurotrophins play a critical role in the growth and stability of NMJs. One neurotrophin, pro-BDNF, is a retrograde trophic factor secreted from muscle fibers in response to presynaptic activity (Misgeld et al., 2002). In its precursor form, proBDNF binds to p75NTR receptors on presynaptic terminals, suppressing neurotransmission and causing synaptic elimination and axonal retraction (Yang et al., 2009). Neuronal activity proteolytically converts proBDNF to mature BDNF (mBDNF), which binds to TrkB receptors to cause synaptic potentiation and neuromuscular maturation. Thus, mBDNf serves as a reward signal to stabilize and maintain the axon terminal during synaptic development and competition (Je et al., 2013; Je et al., 2012). When applied to cultured motoneurons, mBDNF increases axon elongation and growth cone formation, and potentiates calcium transients by increasing Cav2.2 clustering (Dombert et al., 2017). Could low SMN levels result in a deficiency of mBDNF at developing NMJs? If so, what aspect of BDNF signaling is disrupted? It is possible that low SMN levels affects BDNF transcription, translation, or secretion from muscle fibers. Studies using cocultures of rat embryo spinal cord with muscle tissue from SMA patients has demonstrated that muscle fibers were unable to prevent motoneuron death by apoptosis, suggestive of defective neurotrophic function (Guettier-Sigrist et al., 2002). Alternatively, reduced presynaptic transmission may be insufficient to elicit an adequate amount of BDNF release or cleavage, or perhaps a deficiency in presynaptic TrkB receptor expression prevents motor nerves from responding to the presence of mBDNF. More research is necessary to understand whether chronic potentiation of neuromuscular transmission can increase mBDNF

and/or improve presynaptic stability in SMA.

Neuron-specific z^+ agrin is another activity-driven neurotrophin that is responsible for the aggregation of postsynaptic acetylcholine receptors (DeChiara et al., 1996; Gautam et al., 1996). Z^+ agrin is a heparin sulfate proteoglycan that is released in an activity-dependent manner from the neural synaptic basal lamina (Burgess et al., 1999). Boido and colleagues observed a 50% reduction of Z^+ agrin expression in quadriceps of SMNΔ7 mice, and found that administration of NT-1654 (an agrin mimetic that is resistant to proteolytic cleavage) improved fiber diameter, ameliorated neurofilament accumulation and neuromuscular maturation, and increased motor function (Boido et al., 2018). These results suggest that increasing trophic support for neuromuscular synapses is a therapeutic target to improve SMA pathology. What is unknown is if GV-58 + DAP treatment would affect Z^+ agrin levels in SMA.

4.5 The role of SMN in regulating neuromuscular development

It has been difficult to understand the specific function(s) of SMN that are responsible for the primary pathogenesis in neuromuscular synapses when SMN is decreased. It appears that neuromuscular specialization of lower α-motoneurons requires greater or distinct demands of SMN not required by upper α-motoneurons or other cell types. These large neuromuscular synapses undergo tightly regulated temporal development that is uncompromisingly dependent on high SMN expression. What role(s) of SMN are critical for the correct and timely development of neuromuscular junctions? Possibly, inefficient SMN regulation (due to deleteriously low levels) of pre-mRNA splicing directly causes the defective synaptic circuitry apparent at motoneuron somas and NMJs. SMN is responsible for the splicing of many developmentally regulated genes.

For example, Doktor et al (17) found aberrant splicing of CACNA1 genes (from which derive the pore-forming subunit of VGCCs) in SMNΔ7 mice (Doktor et al., 2017). If a calcium deficiency were a primary cause of neuromuscular pathology, would prenatal administration of GV-58 ± 3,4-DAP rescue neuromuscular maturation?

It appears that at least one role of SMN in neuromuscular development is to temporally regulate the transcription of genes and axonal transport of molecules necessary for the maturation of NMJs. It has been widely observed that neuromuscular development in SMA is delayed (both in humans and animal models), suggesting that SMN directly regulates the timing of maturation. In further support of this conclusion, we found that upregulation of SMN via ASO administration to control mice significantly increases the accumulation of synaptic vesicles in neuromuscular terminals and grip strength (Fig. 23), demonstrating that overexpression of SMN accelerates neuromuscular maturation. Defective maturation is thought to cause neuromuscular denervation (Ling et al., 2012), clearly indicating the importance of achieving this developmental step in a timely fashion. If SMN-deficient motoneurons are incapable of satisfying the temporally restricted developmental demand for functional NMJs, then a dying-back process could contribute to motoneuron loss.

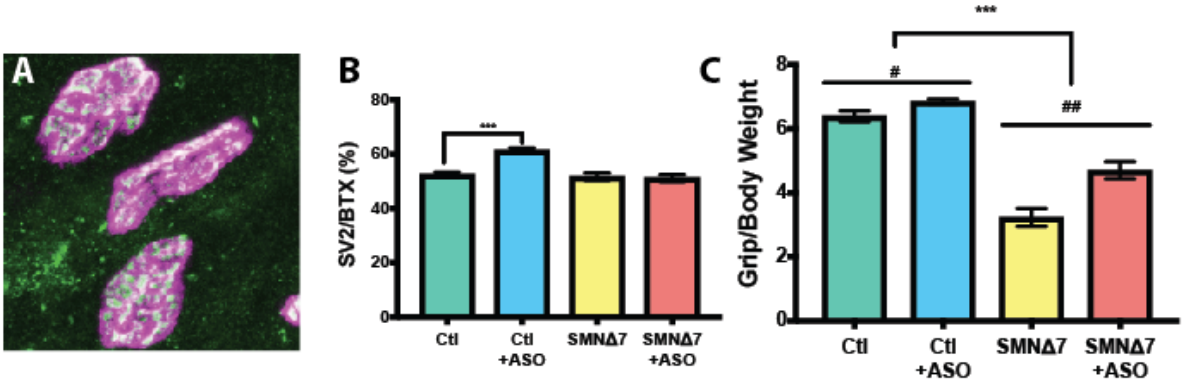

Figure 23 SMN overexpression increases synaptic vesicle pools in motor nerve terminals.

(A) Representative micrograph of SV2 (green) overlaid α-BTX (purple). (B) SV2 (normalized to endplate size) is significantly greater in ASO-treated control mice. (C) Postnatal day 10 ASO-treated control mice are stronger than non-treated control mice (replotted from Figure 17D).

If SMN is generally responsible for the temporal regulation of NMJ development, then why are some muscle-nerve groups resistant to pathology and degeneration? Perhaps some motoneurons require less SMN expression to achieve functional NMJs, or are capable of collateral sprouting to compensate for the loss of a functional synapse. However, it is possible that resistance is not driven solely by compensatory sprouting of motoneurons. Prior to nerve contact, the endplate clustering of acetylcholine receptors is thought to be driven by muscle fibers. We discovered that a mildly vulnerable muscle of SMNΔ7 mice has a significantly greater number of α-BTX-labeled endplates late into the disease progression compared to controls. Could upregulated endplate expression be a compensatory muscle-driven response to the presence of dysfunctional synapses, and is this phenomenon restricted to more resistant muscles? Investigations into the relationship between upregulated endplate expression and motoneuron soma degeneration is needed. Curiously, we measured no effect of ASO treatment on the number of α-BTX labeled synapses in SMNΔ7 mice, suggesting that local factors (given the poor penetrability of ASOs to peripheral tissues) are regulating endplate expression on muscle fibers. It would be interesting to evaluate BDNF or other neurotrophin levels to determine if trophic availability correlates with endplate expression.

4.6 The role of SMN in regulating neuromuscular activity

Neuromuscular maturation is driven by synaptic activity, and reduced neuromuscular transmission has been observed in mild and intermediate SMA mice (Kariya et al., 2008; Kong et al., 2009; Ruiz & Tabares, 2014). Multiple reasons that could cause physiological dysfunction have been discussed (axonal transportation defects, neurofilament blebs, reduced expression and aberrant splicing of VGCCs), but motor nerve terminal excitability has yet to be discussed in depth. Hyperexcitability in SMA mouse motoneurons has been established (Fletcher et al., 2017; Gogliotti et al., 2012; Mentis et al., 2011; Quinlan et al., 2019), but less is known about the excitability of NMJs. In preliminary studies, we observed a change in the shape of the action potential at NMJs of a mildly vulnerable muscle in SMNΔ7 mice during a late stage of disease progression (Fig. 24). Our preliminary evidence using voltage imaging in NMJs suggests that action potential waveforms are about twice as long in duration compared to controls (measured as full width at half maximum; Fig. 24E, F). One reason for this prolonged duration could be a due to insufficient activation of BK channels, which are calcium-activated potassium channels that partially drive action potential repolarization.

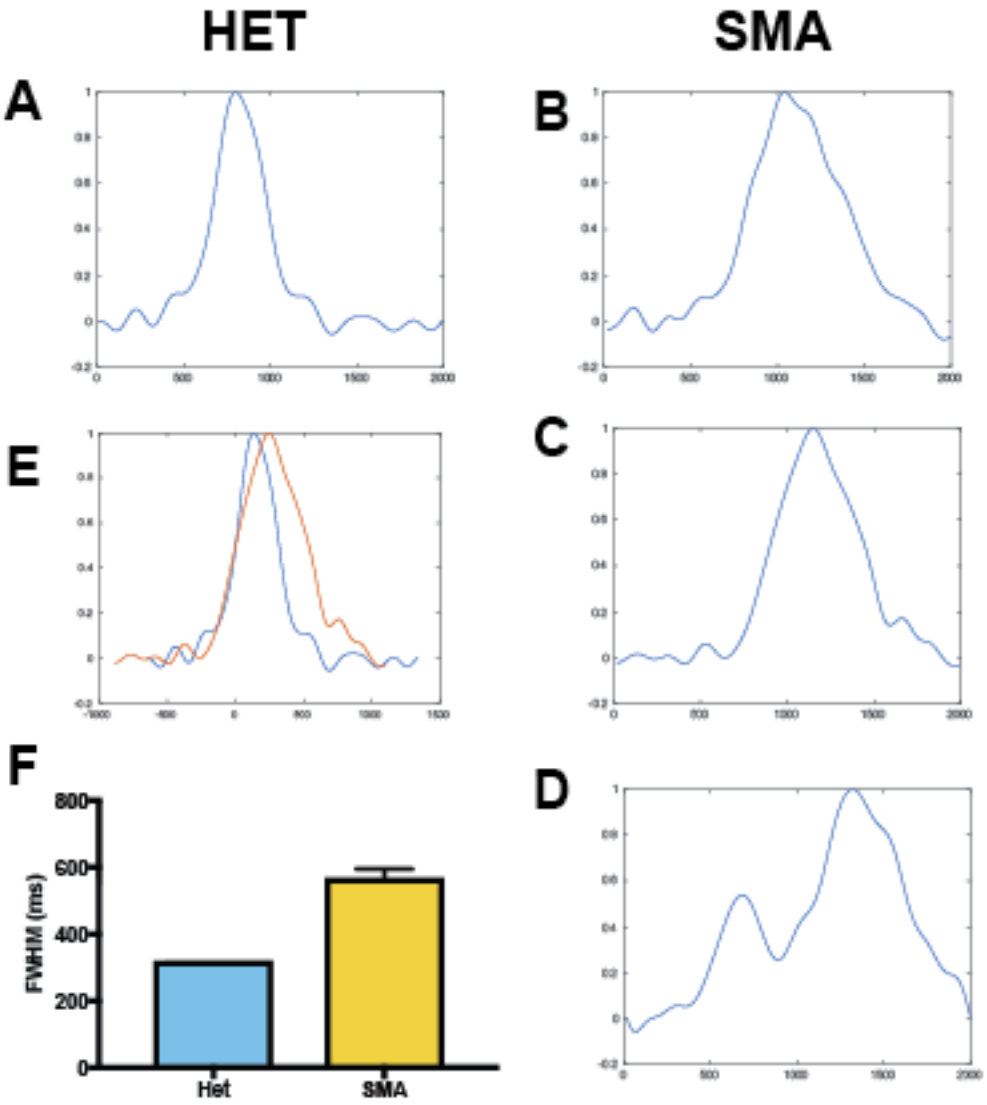

Figure 24 Differences in the action potential duration at NMJs of postnatal day 11 mice.

(A-E) The Y axis represents peak fluorescent signal, and the X axis represents time. In comparison to the heterozygote mouse motor nerve terminal in (A), SMA mouse NMJs (B-D) have nearly twice as long of an action potential duration (E-F). The blue line in (E) represents the heterozygous (het) AP, while the red line indicates the SMA AP. Note the double peak in (D), which shows the delayed maturation in synaptic elimination. Data collected in collaboration with Scott Ginebaugh.

We found slight changes in the action potential rise time, which could be due to aberrant

voltage-gated sodium channel expression (either in density, in splicing variant, or in maturational expression), or possibly due to myelination defects. The largest change in the action potential waveform we observed, however, was in the decay time. Similarly, differences in the expression of voltage-gated potassium channels could contribute to the dramatic delay in AP repolarization. We are also curious about the role of calcium-activated potassium channels in SMA motor terminals. In particular, BK channels are calcium- and voltage-gated potassium channels heavily involved in the repolarization of nerve terminals. Could the observed decay delay be a consequence of these channels failing to activate? Further experimentation is necessary to confirm these observations and to study the role of specific ion channels in the action potential waveform, including effects in SMA.

It is critical to point out the necessary caution required in drawing conclusions from our data. Our preliminary results are based on a limited number of animals (2 SMA mice and 1 heterozygote control). Furthermore, differences in rise and decay times could potentially be artifacts caused by the normalization of the action potential amplitude, since our voltage imaging protocol does not permit the evaluation of membrane voltage. Despite these caveats, it does appear that the duration of action potentials is drastically prolonged in duration in the epitrochleoanconeus muscle of SMNΔ7 mice. Does the action potential waveform also differ in a highly vulnerable muscle? Much remains unknown about the mechanisms underlying potential changes in the action potential waveform, and further investigation is necessary to illuminate any relationship between action potential waveform shape and neuromuscular pathology.

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