Dysregulation of Tweak and Fn14 in skeletal muscle of spinal muscular atrophy mice

Katharina E. Meijboom [1,2], Emma R. Sutton[3], Eve McCallion[3], Emily McFall [4], Daniel Anthony [5], Benjamin Edwards [1], Sabrina Kubinski [6], Ines Tapken [6,7], Ines Bünermann [7], Gareth Hazell [1], Nina Ahlskog [1,8], Peter Claus [6,7], Kay E. Davies [1], Rashmi Kothary [4], Matthew J.A. Wood [1,8], Melissa Bowerman [1,3,9*]

[1] Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom

[2] Gene Therapy Center, UMass Medical School, Worcester, United States.

[3] School of Medicine, Keele University, Staffordshire, United Kingdom.

[4] Ottawa Hospital Research Institute, Regenerative Medicine Program, Ottawa, Canada and; Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Canada.

[5] Department of Pharmacology, University of Oxford, Oxford, United Kingdom.

[6] Institute of Neuroanatomy and Cell Biology, Hannover Medical School, Hannover, Germany and; Center for Systems Neuroscience, Hannover, Germany.

[7] SMATHERIA – Non-Profit Biomedical Research Institute, Hannover, Germany.

[8] Department of Paediatrics, University of Oxford, Oxford, United Kingdom.

[9] Wolfson Centre for Inherited Neuromuscular Disease, RJAH Orthopaedic Hospital, Oswestry, United Kingdom.

[*] Corresponding author: m.bowerman@keele.ac.uk

ABSTRACT

Background: Spinal muscular atrophy (SMA) is a childhood neuromuscular disorder caused by depletion of the survival motor neuron (SMN) protein. SMA is characterized by the selective death of spinal cord motor neurons, leading to progressive muscle wasting. Loss of skeletal muscle in SMA is a combination of denervation-induced muscle atrophy and intrinsic muscle pathologies. Elucidation of the pathways involved is essential to identify the key molecules that contribute to and sustain muscle pathology. The tumor necrosis factor-like weak inducer of apoptosis (TWEAK)/TNF receptor superfamily member fibroblast growth factor inducible 14 (Fn14) pathway has been shown to play a critical role in the regulation of denervation-induced muscle atrophy as well as muscle proliferation, differentiation and metabolism in adults. However, it is not clear whether this pathway would be important in highly dynamic and developing muscle.

Methods: We thus investigated the potential role of the TWEAK/Fn14 pathway in SMA muscle pathology, using the severe Taiwanese *Smn*$^{-/-}$*;SMN2* and the less severe *Smn*$^{2B/-}$ SMA mice, which undergo a progressive neuromuscular decline in the first three post-natal weeks. We also used experimental models of denervation and muscle injury in pre-weaned wild type (WT) animals and siRNA-mediated knockdown in C2C12 muscle cells to conduct additional mechanistic investigations.

Results: Here, we report significantly dysregulated expression of Tweak, Fn14 and previously proposed downstream effectors during disease progression in skeletal muscle of the two SMA mouse models. In addition, siRNA-mediated *Smn* knockdown in C2C12 myoblasts suggests a genetic interaction between Smn and the TWEAK/Fn14 pathway. Further analyses of SMA, *Tweak*$^{-/-}$ and *Fn14*$^{-/-}$ mice revealed dysregulated myopathy, myogenesis and glucose metabolism pathways as a common skeletal muscle feature, providing further evidence in support of a relationship between the TWEAK/Fn14 pathway and Smn. Finally, administration of the TWEAK/Fn14 agonist Fc-TWEAK improved disease phenotypes in the two SMA mouse models.

Conclusions: Our study provides mechanistic insights into potential molecular players that contribute to muscle pathology in SMA and into likely differential responses of the TWEAK/Fn14 pathway in developing muscle.

Keywords: spinal muscular atrophy, survival motor neuron, Smn, Tweak, Fn14, glucose metabolism, skeletal muscle, atrophy, denervation

BACKGROUND

The neuromuscular disease spinal muscular atrophy (SMA) is the leading genetic cause of infant mortality [1]. SMA is caused by mutations in the *survival motor neuron 1* (*SMN1*) gene [2]. The major pathological components of SMA pathogenesis are the selective loss of spinal cord alpha motor neurons and muscle wasting [3]. Skeletal muscle pathology is a clear contributor to SMA disease manifestation and progression and is caused by both denervation-induced muscle atrophy [4] and intrinsic defects [5,6]. As skeletal muscle is the largest insulin-sensitive tissue in the body and is involved in glucose utilization [7], it is not surprising that muscle metabolism is also affected in SMA. Impaired metabolism has indeed been reported in SMA Type 1, 2 and 3 patients [8]. A better understanding of the specific molecular effectors that contribute to SMA muscle physiopathology could provide mechanistic insights in SMA muscle pathology and help therapeutic endeavors aimed at improving muscle health in patients [9].

One pathway that plays a crucial role in chronic injury and muscle diseases is the tumor necrosis factor-like weak inducer of apoptosis (TWEAK) and its main signaling receptor, the TNF receptor superfamily member fibroblast growth factor inducible 14 (Fn14) [10]. TWEAK is ubiquitously expressed and synthesized as a Type II transmembrane protein but can also be cleaved by proteolytic processing and secreted as a soluble cytokine [10]. The role of the TWEAK/Fn14 pathway in skeletal muscle is conflicting as it has been demonstrated to have both beneficial and detrimental effects on muscle health and function [11,12]. Indeed, pathologically high levels of TWEAK activate the canonical nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, which promotes myoblast proliferation and thus inhibits myogenesis and the early phases of muscle repair and regeneration [13]. Conversely, lower physiological concentrations of TWEAK activate the non-canonical NF-κB pathway that promotes myoblast fusion and myogenesis [11]. The transmembrane protein Fn14 is typically dormant or present in low levels in normal healthy muscle [14]. Atrophic-inducing conditions (e.g. casting and surgical denervation) stimulate the expression of Fn14, leading to the chronic activation of the TWEAK/Fn14 pathway and sustained skeletal muscle atrophy [15].

We have also demonstrated an increased activity of the Tweak/Fn14 pathway in skeletal muscle of a mouse model of the neurodegenerative adult disorder amyotrophic lateral sclerosis (ALS), which is characterized by a progressive and chronic denervation-induced muscle atrophy [16]. In addition, various reported downstream effectors of the TWEAK/Fn14 pathway play critical roles in the regulation of muscle metabolism such as peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α), glucose transporter 4 (Glut-4), myogenic transcription factor 2d (Mef2d), hexokinase II (HKII) and Krüppel-like factor 15 (Klf15) [17–20].

Although the TWEAK/Fn14 pathway has been ascribed roles in both skeletal muscle health regulation and metabolism, both of which are impacted in SMA [9,21], this pathway has yet to be investigated in the context of SMA. Furthermore, all research on this pathway has been performed in adult mice and therefore has never been explored in early phases of muscle development. We thus investigated the potential role of TWEAK and Fn14 in SMA and in early phases of post-natal skeletal muscle development. We report significantly decreased levels of both *Tweak* and *Fn14* during disease progression in two distinct SMA mouse models (*Smn*$^{-/-}$*;SMN2* and *Smn*$^{2B/-}$) [22,23]. We also observed dysregulated expression of *PGC-1α, Glut-4, Mef2d* and *HKII*, previously proposed metabolic downstream effectors of TWEAK/Fn14 signaling [18,24], in skeletal muscle of these SMA mice. In addition, more in-depth analyses revealed partial overlap of aberrantly expressed genes that regulate myopathy, myogenesis and glucose metabolism pathways in skeletal muscle of SMA, *Tweak*$^{-/-}$ and *Fn14*$^{-/-}$ mice, further supporting potential shared functions between the TWEAK/Fn14 pathway and SMN in developing muscle. Finally, administration of Fc-TWEAK, an agonist of TWEAK/Fn14 signaling, improved disease phenotypes in the two SMA mouse models. Our study provides additional mechanistic insights into the potential molecular effectors that contribute to skeletal muscle pathology in SMA and suggests a role for the TWEAK/Fn14 pathway in the early stages of post-natal muscle development.

METHODS

Animals and animal procedures

Wild-type mice FVB/N and C57BL/6J and the severe *Smn*$^{-/-}$;*SMN2* mouse model (FVB.Cg-Smn1tm1Hung Tg(SMN2)2Hung/J) [22] were obtained from Jackson Laboratories. The *Smn*$^{2B/-}$ mouse model [23,25] was kindly provided by Dr. Lyndsay M Murray (University of Edinburgh). *Tweak*$^{-/-}$ [26] and *Fn14*$^{-/-}$ mouse models [27] were generously obtained from Linda C. Burkly (Biogen).

Smn$^{-/-}$;*SMN2* and *Smn*$^{+/-}$;*SMN2* mice were generated by breeding *Smn*$^{+/-}$ mice with *Smn*$^{-/-}$;*SMN2/SMN2* mice as previously described [28]. *Smn*$^{2B/-}$ and *Smn*$^{2B/+}$ mice were generated by breeding *Smn*$^{2B/2B}$ and *Smn*$^{+/-}$ mice as previously described [23].

Experimental procedures with live animals were authorized and approved by the University of Oxford ethics committee and UK Home Office (current project license PDFEDC6F0, previous project license 30/2907) in accordance with the Animals (Scientific Procedures) Act 1986, the Keele University Animal Welfare Ethical Review Body and UK Home Office (Project Licence P99AB3B95) in accordance with the Animals (Scientific Procedures) Act 1986, the University of Ottawa Animal Care Committee according to procedures authorized by the Canadian Council on Animal Care and the German Animal Welfare law and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, reference numbers 15/1774 and 19/3309).

Fc-TWEAK was administered by subcutaneous injections using a sterile 0.1 cc insulin syringe at various doses (7.9 μg, 15.8 μg or 31.6 μg) and at a volume of 20 μl either daily, every other day or every four days. Mouse Fc-TWEAK, a fusion protein with the murine IgG2a Fc region, and Ig isotope control were kindly provided by Linda C. Burkly (Biogen) [26].

For survival studies, mice were weighed and monitored daily and culled upon reaching their defined humane endpoint.

For all experiments, litters were randomly assigned at birth and whole litters composed of both sexes were used. Sample sizes were determined based on similar studies with SMA mice.

To reduce the total number of mice used, the fast-twitch tibialis anterior (TA) and triceps muscles from the same mice were used interchangeably for respective molecular and histological analyses.

Sciatic nerve crush and cut

For nerve crush and cut experiments, post-natal day (P) 7 wild-type (WT) FVB/N mice were anesthetized with 2% isoflurane/oxygen before one of their lateral thighs was shaved and a 1 cm incision in the skin was made over the lateral femur. The muscle layers were split with blunt scissors, the sciatic nerve localized and crushed with tweezers for 15 seconds for the nerve crush. For the nerve cut, an ~2 mm section of the nerve was removed and the transection was confirmed under an operating microscope at x12.8. The skin incision was closed with surgical glue and animals allowed to recover on a warming blanket. Ipsilateral and contralateral TA muscles were harvested at P14 and either fixed in 4% paraformaldehyde (PFA) for 24 hours for histological analyses or snap frozen for molecular analyses.

Cardiotoxin injections

Cardiotoxin γ (Cytotoxin I, Latoxan, L8102, Portes les Valence) was dissolved in 0.9% saline and injected 4 µl/g per total mouse weight of a 10 µM solution into the left TA muscle of WT FVB/N mice at post-natal day (P) 10. The right TA was injected with equal volumes of 0.9% saline. During the injection, mice were anesthetized with 2% isoflurane/oxygen and all injections were done using a sterile 0.3 cc insulin syringe. TA muscles were harvested 6 days later and either fixed in 4% PFA for 24 hours for histological analyses or snap frozen for molecular analyses.

Laminin staining of skeletal muscle

TA muscles were fixed in PFA overnight. Tissues were sectioned (13 µm) and incubated in blocking buffer for 2 hours (0.3% Triton-X, 20% fetal bovine serum (FBS) and 20% normal goat serum in PBS). After blocking, tissues were stained overnight at 4°C with rat anti-laminin (1:1000, Sigma L0663) in blocking

buffer. The next day, tissues were washed in PBS and probed using a goat-anti-rat IgG 488 secondary antibody (1:500, Invitrogen A-11006) for one hour. PBS-washed tissues were mounted in Fluoromount-G (Southern Biotech). Images were taken with a DM IRB microscope (Leica) with a 20X objective. Quantitative assays were performed blinded on 3-5 mice for each group and five sections per mouse. The area of muscle fiber within designated regions of the TA muscle sections was measured using Fiji (ImageJ) [29].

Hematoxylin and eosin staining of skeletal muscle

TA muscles were fixated in 4% PFA and imbedded into paraffin blocks. For staining, muscles were sectioned (13 μm) and deparaffinized in xylene and then fixed in 100% ethanol. Following a rinse in water, samples were stained in hematoxylin (Fisher) for 3 minutes, rinsed in water, dipped 40 times in a solution of 0.02% HCl in 70% ethanol and rinsed in water again. The sections were next stained in a 1% eosin solution (BDH) for 1 minute, dehydrated in ethanol, cleared in xylene, and mounted with Fluoromount-G (Southern Biotech). Images were taken with a DM IRB microscope (Leica) with a 20X objective. Quantitative assays were performed blinded on 3-5 mice for each group and five sections per mouse. The area of muscle fibre within designated regions of the TA muscle sections was measured using Fiji (ImageJ) [29].

Cell culture

Both C2C12 myoblasts [30] and NSC-34 neuronal-like cells [31] were maintained in growth media consisting of Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% FBS and 1% Penicillin/Streptomycin (all Life Technologies). Cells were cultured at 37°C with 5% CO_2. C2C12 myoblasts were differentiated in DMEM containing 2% horse serum for 7 days to form multinucleated myotubes.
Cells were regularly tested for mycoplasma and remained mycoplasma-free.

In vitro siRNA knockdown

For small interfering RNA (siRNA) transfections, C2C12 myoblasts were seeded onto 12-well plates at a 50% confluency and cultured overnight in 2 mL of DMEM. Cells were washed with PBS prior to siRNA transfection, whereby 100 pmol of each siRNA (*Tweak*, *Fn14*, *Smn*) (Invitrogen, assay IDs s233937, s203164, s74017, respectively) in a complex with 10 µl of Lipofectamine RNAi/MAX (Invitrogen) dissolved in OptiMEM solution (Gibco) was added to the cells for three hours. The transfection mix was then substituted either for DMEM without the siRNAs for 1 day or with a differentiation medium mix without the siRNAs for 7 days.

qPCR

RNA was extracted from tissues and cells either by a RNeasy kit from Qiagen or by a Isolate II RNA Mini Kit from Bioline or by guanidinium thiocyantate-acid-phenol-chloroform extraction using TRIzol Reagent (Life Technologies) as per manufacturer's instructions. The same RNA extraction method was employed for similar experiments and equal RNA amounts were used between samples within the same experiments. cDNA was prepared with the High Capacity cDNA Kit (Life Technologies) or qPCRBIO cDNA Synthesis Kit (PBCR Biosystems) according to the manufacturer's instructions. The same reverse transcription method was employed for similar experiments. The cDNA template was amplified on a StepOnePlus Real-Time PCR Thermocycler (Life Technologies) with SYBR Green Mastermix from Applied Biosystems or with qPCRBIO SyGreen Blue Mix Hi-ROX (PCR Biosystems). The same amplification method was used for similar experiments. qPCR data was analyzed using the StepOne Software v2.3 (Applied Biosystems). Primers used for qPCR were obtained from IDT and sequences for primers were either self-designed or ready-made (Supplementary Table 1). Relative gene expression was quantified using the Pfaffl method [32] and primer efficiencies were calculated with the LinRegPCR software. We normalized relative expression level of all tested genes in mouse tissue and cells to *RNA polymerase II polypeptide J* (*PolJ*) [33]. For all qPCR graphs, the normalized expression of the experimental groups is compared to a referent group, for which the normalized expression values were set to 1 by multiplying the normalized expression of each

referent sample in that group by the value corresponding to 1/(Average of all samples in that referent experimental group). That value was then used to multiply the normalized relative expression of each sample in all experimental groups.

PCR array

RNA was extracted using the RNeasy® Microarray Tissue Kit (Qiagen). cDNA was generated with the RT^2 First Strand Kit (Qiagen). qPCRs were performed using RT² Profiler™ PCR Array Mouse Skeletal Muscle: Myogenesis & Myopathy Mouse (PAMM-099Z, SABiosciences) and RT² Profiler™ PCR Array Mouse Glucose Metabolism (PAMM-006Z SABiosciences). The data were analyzed with RT Profiler PCR Array Data Analysis (version 3.5) and mRNA expression was normalized to the two most stably expressed genes between all samples. We used the publicly available database STRING (version 10.5) for network and enrichment analysis of differently expressed genes [34]. The minimum required interaction score was set at 0.4, medium confidence.

Western blot

For westerns in Figure 1, freshly prepared radioimmunoprecipitation (RIPA) buffer was used to homogenize tissue and cells, consisting of 50 mM Tris pH 8.8, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and complete mini-proteinase inhibitors (Roche). Equal amounts of total protein were loaded, as measured by Bradford Assay. Protein samples were first diluted 1:1 with Laemmli sample buffer (Bio-Rad, Hemel Hempstead, UK) containing 5% β-mercaptoethanol (Sigma) and heated at 100°C for 10 minutes. Next, samples were loaded on freshly made 1.5 mm 12% polyacrylamide separating and 5% stacking gel and electrophoresis was performed at 120 V for ~1.5 hours in running buffer. Subsequently, proteins were transferred from the gel onto to a polyvinylidene fluoride membrane (Merck Millipore) via electroblotting at 120 V for 60 minutes in transfer buffer containing 20% methanol. Membranes were then incubated for 2 hours in Odyssey Blocking Buffer (Licor). The membrane was then probed overnight at 4°C with primary

antibodies (P105/p50, 1:1000, Abcam ab32360; Actin, 1:1000, Abcam ab3280) in Odyssey Blocking Buffer and 0.1% Tween-20. The next day, after three 10-minute washing steps with PBS, the membrane was incubated for 1 hour at room temperature with secondary antibodies (goat anti-rabbit IgG 680RD, 1:1000, LI-COR 926-68071; goat anti-mouse IgG 800CW, 1:1000 LI-COR, 926-32210). Lastly, the membrane was washed three times for 10 minutes in PBS and visualized by scanning 700 nm and 800 nm channels on the LI-COR Odyssey CLx infrared imaging system (LI-COR) for 2.5 minutes per channel. The background was subtracted and signal of protein of interest was divided by signal of the housekeeping protein.

For westerns in all others figures, the same steps were followed with the following key differences. Bio-Rad TGX Stain-Free gels were used and gels were imaged on a Chemi-Doc Bio-Rad Imager before transfer to quantify total protein used for normalization. The primary antibodies used were NF-κB2 p100/p52 (Cell Signaling, #4882, 1:1000), NF-κB1 p105/p50 (Cell Signaling, #12540, 1:1000) and Tweak (Abcam, ab37170, 1:1000). The secondary antibody used was goat anti-rabbit IgG secondary Dylight 800 (Invitrogen, SA5-100036, 1:10000). Quantification was performed using the Bio-Rad Image Lab Software.

Statistical Analysis

All statistical analyses were done with the most up to date GraphPad Prism software. When appropriate, a Student's unpaired two-tail *t*-test, a one-way ANOVA or a two-way ANOVA was used. *Post-hoc* analyses used are specified in Figure Legends. Outliers were identified via the Grubbs' test. For the Kaplan-Meier survival analysis, the log-rank test was used and survival curves were considered significantly different at $p<0.05$.

RESULTS

Tweak and Fn14 are dysregulated in two SMA mouse models

We firstly investigated the expression of Tweak and Fn14 in skeletal muscle of the severe Taiwanese *Smn*$^{-/-}$;*SMN2* mouse model [22], using muscles with reported differential vulnerability to neuromuscular junction (NMJ) denervation (vulnerability: triceps brachii > gastrocnemius > TA > quadriceps femoris) [35]. Muscles were harvested from *Smn*$^{-/-}$;*SMN2* and WT mice at several time points during disease progression: birth (post-natal day (P) 0, pre-symptomatic (P2), early symptomatic (P5), late-symptomatic (P7) and end stage (P10)). Muscle pathology in this SMA mouse model during disease progression has been well documented [36,37].

We assessed the expression of *parvalbumin*, a high affinity Ca^{2+}-binding protein, which is downregulated in denervated muscle [38,39] and a marker of muscle atrophy in skeletal muscle of SMA patients and *Smn*$^{-/-}$;*SMN2* mice [40]. We observed a significant decreased expression of *parvalbumin* mRNA during disease progression (Fig. 1a) in SMA mice compared to WT animals, further confirming parvalbumin as a *bona fide* marker of muscle atrophy in SMA [40]. Furthermore, we noted that parvalbumin expression was downregulated at earlier time points in the two most vulnerable muscles (triceps and gastrocnemius) [35] of SMA mice compared to WT animals (Fig. 1a).

We next evaluated the expression of *Tweak* and *Fn14* and observed significant decreased levels of *Tweak* mRNA in muscles of *Smn*$^{-/-}$*;SMN2* mice during disease progression, except in the quadriceps (Fig. 1b). Similarly, we found significantly lower levels of *Fn14* mRNA in all muscles of *Smn*$^{-/-}$*;SMN2* mice during disease progression (Fig. 1c) compared to WT animals. Interestingly, the decreased expression of *Fn14* in denervated and atrophied muscles of neonatal animals is different to previous reports in adults where denervation-induced atrophy stimulates its expression [15,16].

As mentioned above, the TWEAK/Fn14 pathway has been reported to negatively influence the expression of metabolic effectors Klf15, Pgc-1α, Mef2d, Glut-4 and HKII [18]. Given that we have previously published a concordant increased expression of *Klf15* in skeletal muscle of SMA mice during disease progression [41], we next evaluated if the additional metabolic targets proposed to be modulated by Tweak and Fn14 were similarly dysregulated in the predicted directions. We indeed observed that the mRNA expression of *Pgc-1α*, *Mef2d*, *Glut-4* and *HKII* was significantly upregulated in muscles of *Smn*$^{-/-}$*;SMN2* mice at symptomatic time-points (P5-P10) compared to WT animals (Fig. 1d-g), showing an expected opposite pattern to both *Tweak* and *Fn14* (Fig. 1b-c) [18]. Notably, we also found that in most muscles, mRNA levels of *Pgc-1α*, *Mef2d*, *Glut4* and *HKII* were significantly decreased in pre-symptomatic *Smn*$^{-/-}$*;SMN2* mice (P0-P5) compared to WT animals (Fig. 1d-g), independently of *Tweak* and *Fn14* (Fig. 1b-c).

TWEAK and Fn14 have also been reported to impact the canonical and non-canonical NF-κB pathways in skeletal muscle [42,43]. In pre-symptomatic (P2) TA muscle, we observed no significant difference in the expression of NF-κB1 (p50), a component of the canonical NF-κB pathway, between *Smn*$^{-/-}$;*SMN2* mice and WT animals (Fig. 1h), consistent with normal *Tweak* and *Fn14* levels (Fig. 1b-c). Conversely, there was a significant decreased expression of NF-κB1 (p50) in TA muscle of symptomatic *Smn*$^{-/-}$;*SMN2* mice compared to WT animals at P7 (Fig. 1i), in line with reduced levels of *Tweak* and *Fn14* (Fig. 1b). These findings are validated in P7 quadriceps, where NF-κB1 (p50) levels are also significantly decreased in *Smn*$^{-/-}$*;SMN2* mice compared to WT animals (Fig. 1j). We found no significant difference for the p105 NF-κB1 component. Of note, for all NF-κB1 p50/105 westerns, the p105 component was always more difficult to detect and sometimes even undetectable such as was the case for P7 TAs. We also investigated the expression of NF-κB-inducing kinase (NIK), involved in the non-canonical NF-κB activation pathway [44]. We observed that mRNA levels of *NIK* were significantly increased in TA muscle of P7 *Smn*$^{-/-}$;*SMN2* mice compared to WT animals (Fig. 1k), suggesting that dysregulated activity of Tweak and Fn14 in skeletal

muscle of SMA mice may influence both the canonical and non-canonical NF-κB pathways, which play key regulatory roles in muscle health and metabolism [11,12].

Finally, we evaluated the expression of Tweak and Fn14 in skeletal muscle of the less severe *Smn*$^{2B/-}$ mouse model of SMA [23]. TA muscles were harvested from *Smn*$^{2B/-}$ mice and age-matched WT animals at P0 (birth), P2 (early pre-symptomatic), P4 (late pre-symptomatic), P11 (early symptomatic) and P19 (end stage). Similar to the *Smn*$^{-/-}$*;SMN2* mice, muscle pathology in this SMA mouse model during disease progression has been well documented [36,37]. We found a significant decreased expression of *parvalbumin* (Fig. 2a), *Tweak* (Fig. 2b) and *Fn14* (Fig. 2c) in muscle from *Smn*$^{2B/-}$ mice during disease progression compared to WT animals, similar to that observed in the more severe *Smn*$^{-/-}$*;SMN2* SMA mouse model (Fig. 1a-c). We have previously reported the aberrant increased expression of *Klf15* in the TA muscle of *Smn*$^{2B/-}$ mice during disease progression [41]. However, *Pgc-1α* expression was increased at P11 only (Fig. 2d), *Mef2d* at P2 only (Fig. 2e), *Glut-4* at P11 only (Fig. 2f), while *HKII* was significantly decreased at P0 and P19 and significantly increased at P4 (Fig. 2g), suggesting that the proposed negative impact of Tweak and Fn14 activity on these metabolic effectors may be dependent on disease severity, age and/or genetic strain. Tweak downregulation in triceps of P18 *Smn*$^{2B/-}$ mice was confirmed by western (Fig. 2h). Furthermore, contrary to what was observed in the *Smn*$^{-/-}$*;SMN2* mice, there was no significant difference in the NF-κB1 p50 component but a significant decreased expression of the NF-κB1 p105 component in skeletal muscle of *Smn*$^{2B/-}$ mice compared to WT animals (Fig. 2i). For the NF-κB2 pathway, we found no significant difference for either the p52 or the p100 components (Fig. 2j). Thus, our results point to distinct profiles of the NF-κB1 and 2 pathways in skeletal muscle of the two SMA mouse models, which could be due to differential expression and/or processing of the components and to non-Tweak/Fn14 pathways.

To determine if the dysregulated expression of Tweak, Fn14 and the previously reported metabolic effectors in SMA muscle is independent of disease status, we investigated the mRNA expression of *Tweak*, *Fn14*,

Pgc-1α, *Mef2d*, *Glut-4*, *HKII* and *Klf15* in triceps of P7 WT, $Smn^{2B/2B}$ and $Smn^{+/-}$ mice (Supplementary Fig. 1), a time-point at which significant changes were already observed in the $Smn^{-/-}$;*SMN2* mice. $Smn^{2B/2B}$ and $Smn^{+/-}$ mice express ~70% and 50% of full-length functional Smn protein compared to WT animals, respectively, and do not display a canonical SMA phenotype [23,45]. While we found some instances of differential expression (*Glut-4*: $Smn^{2B/2B}$ vs $Smn^{+/-}$; *HKII*: $Smn^{2B/2B}$ vs $Smn^{+/-}$ and *Klf15*: WT vs $Smn^{+/-}$), there is no clear correlation between non-pathological Smn levels (WT vs $Smn^{2B/2B}$ vs $Smn^{+/-}$) and expression of molecular components associated with the Tweak/Fn14 pathway (Supplementary Fig. 1).

We have thus demonstrated that Tweak, Fn14 and associated metabolic effectors are dysregulated during progressive muscle atrophy in two SMA mouse models and that this is most likely due to pathological levels of Smn depletion.

Denervation does not affect Tweak and Fn14 during the early stages of muscle development

As SMA muscle pathology is defined by both intrinsic defects and denervation-induced events, we set out to determine which of these may influence the dysregulation of Tweak and Fn14 in SMA muscle. We firstly addressed the denervation component by performing nerve crush experiments in which the sciatic nerves of P7 WT mice were crushed and the muscle harvested at P14 [46]. Of note, the sciatic nerve was crushed in only one hindlimb, leaving the other control hindlimb intact. Quantification of myofiber area in TA muscles showed a significant decrease in myofiber size in the nerve crush muscle compared to the control hindlimb (Fig. 3a-c).

Expression analyses further revealed that there were no significant changes in mRNA levels of *parvalbumin*, *Tweak*, *Fn14*, *PGC-1α*, *Mefd2*, *Glut-4* and *HKII* in the denervated muscle compared to the control TA muscle (Fig. 3d). Interestingly, while denervation in adult muscle has previously been reported to induce a dramatic surge in Fn14 expression [15,16], this did not occur in the denervated muscles of our pre-weaned

mice, suggesting an age and/or development regulatory element to this response. We also investigated the expression of *Klf15* and *Smn* and similarly observed no significant differences between the nerve crush and control muscles (Fig. 3d). To ensure that our results were not influenced by the potential reinnervation of muscles following a nerve crush, we repeated the experiments by performing a nerve cut instead. We observed that this complete denervation of TAs in pre-weaned mice does not significantly impact the mRNA expression of *Tweak*, *Fn14*, *PGC-1α*, *Mefd2*, *Glut-4*, *HKII* and *Klf15* compared to uninjured control hindlimbs (Supplementary Fig. 2).

Overall, these results suggest that the dysregulation of parvalbumin, Tweak, Fn14 and the proposed metabolic effectors in SMA muscle during disease progression is most likely not denervation-dependent.

Intrinsic muscle injury affects Tweak and Fn14 during the early stages of muscle development

We next investigated what impact impairing intrinsic muscle integrity would have on Tweak and Fn14. To do so, we used cardiotoxin to induce myofiber necrosis. Cardiotoxin was injected in P10 WT mice into the left TA while the right TA was injected with equal volumes of 0.9% saline and used as a control. TAs were harvested after 6 days, a time-point where muscles are still in an immature and regenerating mode [47]. Indeed, analysis of centrally located nuclei showed a significantly increased percentage of regenerating myofibers in cardiotoxin-treated muscles compared to saline-treated TAs (Fig. 4a-b).

We then proceeded with molecular analyses and observed that the atrophy marker *parvalbumin* was significantly downregulated in cardiotoxin-treated TA muscles compared to saline-treated TA muscles (Fig. 4c). *Fn14* mRNA expression was significantly increased after cardiotoxin injury (Fig. 4c), in accordance with previous research showing that muscle damage conditions activate Fn14 [15]. Conversely, *Pgc-1α*, *Glut-4*, *HKII* and *Klf15* mRNA levels were significantly downregulated (Fig. 4c), supporting their previously reported negative response to active Tweak and Fn14 [18]. Interestingly, *Tweak* mRNA expression remained

unchanged (Fig. 4c), contrary to previous reports of upregulation following cardiotoxin injury in adult muscle [48], suggesting a differential response in early developmental stages of skeletal muscle. Notably, *Smn* expression was significantly increased in the regenerating muscles compared to saline-treated TA muscles (Fig. 4c), perhaps due to SMN's reported role during muscle fiber regeneration [49].

Together, these results suggest that intrinsic muscle injury in pre-weaned mice induces a dysregulation of Tweak, Fn14 and previously reported proposed metabolic effectors. However, the changes were in the opposite direction than that observed in SMA muscles (Fig. 1b), perhaps due to the necrosis and regeneration events that occur following cardiotoxin injury [50], which are not typically found in muscles of SMA mice.

Genetic interactions between Smn, Tweak and Fn14 in muscle

We next wanted to further understand the potential relationship between dysregulated expression of *Tweak*, *Fn14* and *Smn* in skeletal muscle of SMA mice. To do so, we evaluated the impact of Tweak and Fn14 depletion in the early stages of muscle development by performing molecular analyses on P7 triceps from *Fn14*$^{-/-}$, *Tweak*$^{-/-}$ and WT mice. In *Tweak*$^{-/-}$ mice, we observed a significant increased expression of *Fn14* with a concomitant significantly decreased expression of *Klf15* compared to WT animals (Fig. 5a). Notably, we found a significant decreased expression of *Smn* in *Tweak*$^{-/-}$ triceps compared to WT mice (Fig. 5a), suggesting a direct or indirect positive interaction between Tweak and Smn levels. For their part, *Fn14*$^{-/-}$mice displayed a significant downregulation of *parvalbumin* and a significant upregulation of *Pgc-1α* (Fig. 5b). These analyses further support the previously reported negative influence of Fn14 on Pgc-1α and Klf15 expression as well as the absence of overt pathological muscle phenotypes in young *Tweak*$^{-/-}$ and *Fn14*$^{-/-}$ mice [15,51].

To further dissect the relationship between Smn, Tweak and Fn14 during myogenic differentiation, we performed siRNA-mediated knockdown of *Smn*, *Tweak* and *Fn14* in C2C12 myoblasts and evaluated the

effect on the expression of Tweak, Fn14 and the previously reported proposed metabolic effectors in undifferentiated (Day 0) and differentiated (Day 7) cells. Reduced levels of *Smn*, *Tweak* and *Fn14* were significantly maintained in both proliferating and differentiated cells following transfection with *siSmn*, *siTweak* and *siFn14*, respectively (Fig. 5c-e). We observed an interaction between *Smn*, *Tweak* and *Fn14* specifically in differentiated C2C12s, whereby *Smn* expression was significantly upregulated in *Fn14*-depleted D7 cells (Fig. 5c), *Tweak* expression was significantly reduced in *Smn*-depleted D7 cells (Fig. 5d), and *Fn14* levels were significantly decreased in *Tweak*- and *Smn*-depleted D7 cells (Fig. 5e). Similarly, the effects of siRNA-mediated knockdown of *Smn*, *Tweak* and *Fn14* on the metabolic effectors were only apparent in differentiated C2C12s (Fig. 5f-j). Indeed, both knockdown of *Tweak* and *Fn14* resulted in a significant upregulation of *Pgc-1α* (Fig. 5f) and *Mef2d* (Fig. 5g). While *Glut-4* expression was neither affected by depletion of *Smn*, *Tweak* or *Fn14* (Fig. 5h), *HKII* mRNA levels were significantly decreased following knockdown of all three (Fig. 5i). Finally, *Klf15* expression was significantly increased in siRNA-mediated knockdown of *Fn14* only (Fig. 5j). The upregulation of *Pgc-1α*, *Mef2d*, and *Klf15* in *Tweak*- and/or *Fn14*-depleted differentiated C2C12 cells is in accordance with the previously reported downregulation of these genes when Tweak and Fn14 are active, while the unchanged *Glut-4* and downregulated *HKII* levels were not [52].

Thus, using both *in vivo* and *in vitro* models, we have thus provided evidence for a potential interaction between *Smn*, *Tweak* and *Fn14* and subsequent impact on the previously proposed downstream metabolic effectors (Fig. 5k). Our results suggest that the aberrant expression of Tweak and Fn14 in SMA muscle during disease progression may be due to a dynamic interplay between muscle-specific conditions and the molecular impact, individual and combined, of reduced expression of Smn, Tweak and Fn14 in the early developmental stages of skeletal muscle.

Overlap of dysregulated myopathy and myogenesis genes and glucose metabolism genes in *SMA*, *Fn14*$^{-/-}$ and *Tweak*$^{-/-}$ mice

To further decipher the potential contribution(s) of Smn, Tweak and Fn14 depletion to SMA muscle pathology, we used commercially available mouse myopathy and myogenesis qPCR arrays (SABiosciences), which measure expression levels of a subset of 84 genes known to display and/or regulate myopathy and myogenesis. We used triceps (vulnerable) and quadriceps (resistant) from P7 *Smn*$^{-/-}$;*SMN2*, *Tweak*$^{-/-}$, *Fn14* $^{-/-}$ mice. WT FVB/N mice were compared to SMA animals and WT C57BL/6 mice were compared to *Tweak*$^{-/-}$ and *Fn14*$^{-/-}$ mice to account for differences due to genetic strains. Unsurprisingly, we observed a larger number of significantly dysregulated myopathy and myogenesis genes in triceps of *Smn*$^{-/-}$;*SMN2* mice than in the more resistant quadriceps, some of which overlapped with the subset of genes aberrantly expressed in *Fn14*$^{-/-}$ mice and *Tweak*$^{-/-}$ mice (Fig. 6a, Table 1, Supplementary File 1). We also used the publicly available database STRING [34] to perform network and enrichment analysis of the shared differentially expressed genes in both triceps and quadriceps (Table 1), which revealed that there were no known protein-protein interactions between any of the dysregulated genes and Smn, Fn14 or Tweak (Fig. 6b). Interestingly, the central connectors *Myod1* and *Myf6* were upregulated in *Tweak*$^{-/-}$ and *Fn14*$^{-/-}$ mice and *Pax7* was downregulated in the triceps of all three experimental groups (Table 1). Myod1 and Myf6 are key myogenic regulatory factors (MRFs) and are normally upregulated after skeletal muscle injury [53]. Pax7 is a canonical marker for satellite cells, the resident skeletal muscle stem cells [53], and reduced activity of Pax7 leads to cell-cycle arrest of satellite cells and dysregulation of MRFs in skeletal muscle [54]. Furthermore, *Titin* (*Ttn*) was downregulated in the quadriceps muscles of all three mouse models and plays major roles in muscle contraction and force production, highlighted by titin mutations leading to a range of skeletal muscle diseases and phenotypes [55].

Next, as SMN, TWEAK and Fn14 have been associated with glucose metabolism abnormalities [18,56], we performed similar gene expression analyses with commercially available qPCR arrays (SABiosciences)

containing a subset of 84 genes known to display and/or regulate glucose metabolism. We found a similar large number of genes that were dysregulated in both triceps and quadriceps muscles of *Smn*$^{-/-}$;*SMN2* mice, some of which overlapped with those differentially expressed in *Fn14*$^{-/-}$ and *Tweak* $^{-/-}$ mice (Fig. 6c, Table 2, Supplementary File 2). STRING network and enrichment analysis [34] revealed that there are no known protein-protein interactions between any of the dysregulated genes and Smn, Fn14 or Tweak (Fig. 6d). Further analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways composed of the glucose metabolism genes significantly dysregulated in the same direction in triceps and quadriceps muscles of P7 *Smn*$^{-/-}$;*SMN2, Fn14*$^{-/-}$ and *Tweak*$^{-/-}$ mice as well as the downstream effectors of the TWEAK/Fn14 pathway studied in this project (Pgc-1α, Mef2d, Glut4, Klf15, and HKII) reveals that many aspects of glucose metabolism such as insulin signaling, glycolysis are dysregulated in Smn-, Tweak- and Fn14-depleted mice (Table 3).

We thus show a shared pattern of aberrantly expressed genes that modulate myogenesis, myopathy and glucose metabolism in SMA, Tweak-depleted and Fn14-depleted skeletal muscle, suggesting that Smn, Tweak and Fn14 may act synergistically on muscle pathology and metabolism defects in SMA muscle.

Administration of the Fc-TWEAK agonist improves a subset of disease phenotypes in two SMA mouse models

Finally, we evaluated the impact of increasing Tweak activity on disease progression and muscle pathology in SMA mice.

Of note, while the *Smn*$^{+/-}$*;SMN2* and *Smn*$^{2B/+}$ mice are healthy littermates in terms of lifespan and reproductive abilities, they nevertheless have reduced levels of Smn, which in itself has been demonstrated to impact certain phenotypic features (e.g. tail and ear necrosis, metabolism, gene expression). As such, and similar to previous studies [41], comparisons were performed between untreated and Fc-TWEAK-treated

475 animals of the same genotype, allowing us to determine if the effects were SMA-dependent and/or -
476 independent, without the addition of a potential compounding factor.
477
478 Firstly, *Smn*$^{-/-}$*;SMN2* mice and healthy littermates received a daily subcutaneous injection of Fc-TWEAK
479 (15.8 µg), a fusion protein with the murine IgG2a Fc region [26], starting at birth. We found that Fc-TWEAK
480 did not significantly impact weight or survival of *Smn*$^{-/-}$*;SMN2* mice compared to untreated and IgG-treated
481 controls (Fig. 7a-b). Additional lower (7.9 µg) and higher doses (23 and 31.6 µg) were also administered but
482 proved to negatively impact weight and survival (Supplementary Fig. 3).
483
484 Triceps from P7 untreated and Fc-TWEAK-treated (15.8 µg) *Smn*$^{-/-}$;*SMN2* SMA mice and *Smn*$^{+/-}$*;SMN2*
485 healthy littermates were further processed for molecular analyses of the Tweak/Fn14 pathway. We observed
486 that Fc-TWEAK administration did not influence the expression of *Tweak* (Fig. 7c) or *Fn14* (Fig. 7d) in
487 neither *Smn*$^{+/-}$*;SMN2* nor *Smn*$^{-/-}$;*SMN2* mice compared to untreated animals. Similarly, Fc-TWEAK did not
488 induce changes in *Pgc-1α* expression (Fig. 7e). We did observe a significant downregulation of *Mef2d* in
489 Fc-TWEAK-treated muscles of *Smn*$^{-/-}$*;SMN2* SMA mice compared to untreated animals (Fig. 7f). *Glut-4*
490 mRNA expression remained unchanged in both *Smn*$^{+/-}$*;SMN2* and *Smn*$^{-/-}$;*SMN2* Fc-TWEAK-treated mice
491 (Fig. 7g). *HKII* was significantly upregulated in muscle of Fc-TWEAK-treated *Smn*$^{+/-}$*;SMN2* healthy
492 littermates while it was significantly downregulated in Fc-TWEAK-treated *Smn*$^{-/-}$*;SMN2* SMA mice
493 compared to untreated groups (Fig. 7h). *Klf15* was significantly downregulated in Fc-TWEAK-treated *Smn*$^{-/-}$*;SMN2* SMA mice only compared to untreated SMA animals (Fig. 7i). The absence of overt changes in the
495 expression of Tweak, Fn14 and the previously reported proposed downstream metabolic effectors may be
496 due to the 24-hour time-lapse between the last Fc-TWEAK injection and harvest of tissues, which could
497 have led to missing key time-points at which transcriptional profiles were significantly impacted.
498

Whilst we did not capture the short-term molecular effects of Fc-TWEAK administration, quantification of myofiber area in TA muscles showed that daily Fc-TWEAK treatment significantly increased myofiber area in skeletal muscle of P7 *Smn*$^{-/-}$;*SMN2* mice compared to untreated SMA animals (Fig. 7j-k). Furthermore, the expression of atrophy markers *parvalbumin*, *MuRF-1* and *atrogin-1* [57] was also restored towards normal levels, whereby *parvalbumin* expression was significantly increased (Fig. 7l) whilst *MuRF-1* and *atrogin-1* expression was significantly downregulated (Fig. 7m-n) in triceps of Fc-TWEAK-treated *Smn*$^{-/-}$*;SMN2* SMA mice compared to untreated SMA animals, further supporting an improvement in muscle health. We did not however detect changes in MRFs *Myod1* and *myogenin* [53] (Fig. 7o-p).

We next assessed the effect of Fc-TWEAK in *Smn*$^{2B/-}$ mice, which are typically more responsive to Smn-independent treatment strategies [41,58–60]. Due to the longer treatment period in these mice (20 days) and the observed toxicity in daily injected mice (> 10 days), the *Smn*$^{2B/-}$ and *Smn*$^{2B/+}$ mice received subcutaneous injections of Fc-TWEAK and IgG control (15.8 µg) every 4 days, starting at birth. Both IgG and Fc-TWEAK did not significantly impact the weight of *Smn*$^{2B/-}$ mice compared to untreated SMA animals (Fig. 7q). However, Fc-TWEAK significantly increased the lifespan of *Smn*$^{2B/–}$ mice compared to both IgG-treated and untreated animals (Fig. 7r). Molecular analyses of the mRNA levels of *Tweak*, *Fn14* and the previously reported proposed molecular effectors in triceps from P15 animals only showed a significant effect of Fc-TWEAK on the expression of *Glut-4*, whereby it was downregulated in Fc-TWEAK-treated *Smn*$^{2B/-}$ mice compared to untreated animals (Fig. 7s). Similarly to above, the limited impact of Fc-TWEAK on the expression of Tweak, Fn14 and the previously reported metabolic effectors in P15 animals may be due to the 72-hour time-lapse between the last injection of Fc-TWEAK and tissue harvest. This experimental paradigm was chosen to follow the optimal dosing regimen and perform molecular analyses at a symptomatic time-point that was too close to the end stage of the disease. Nevertheless, to determine if molecular changes could be captured following a shorter time-lapse between the Fc-TWEAK injection and tissue harvest and to determine if the response to Fc-TWEAK is different in WT tissues that express 100% Smn, we treated

WT and *Smn*$^{2B/-}$ with Fc-TWEAK (15.8 ug) every 4 days from birth until P16 and harvested skeletal muscle 3 hours post-injection. While *Fn14* mRNA expression remained unchanged in the triceps from both WT and SMA mice (Supplementary Fig. 4a), we found differential expression patterns of the other metabolic effectors proposed to be influenced by Tweak and Fn14. Indeed, following Fc-TWEAK injections, *Tweak* mRNA is significantly increased in WT animals and unchanged in *Smn*$^{2B/-}$ mice (Supplementary Fig. 4b), *PGC-1a* and *Mef2d* are unchanged in WT animals and significantly decreased in *Smn*$^{2B/-}$ mice (Supplementary Fig. 4c-d), while *Glut-4*, *HKII* and *Klf15* are significantly increased in WT animals and significantly decreased in *Smn*$^{2B/-}$ animals (Supplementary Fig. 4e-g). Similarly, we observed a specific decrease of the NF-κB2 p100 component (all other components were unchanged) in Fc-TWEAK-treated WT animals compared to untreated controls while it is significantly upregulated in Fc-TWEAK-treated *Smn*$^{2B/-}$ mice compared to untreated animals (Supplementary Fig. 4h).

As improvements in muscle health parameters were observed in Fc-TWEAK-treated *Smn*$^{-/-}$*;SMN2* SMA mice, we performed similar investigations in *Smn*$^{2B/-}$ mice. Contrary to the more severe mouse model, we did not find any significant changes in expression levels of *parvalbumin*, *MuRF-1*, *atrogin-1* and *myod1* in neither *Smn*$^{2B/+}$ or *Smn*$^{2B/-}$ Fc-TWEAK-treated animals (Fig. 7t-w). We did observe a significant increase in *myogenin* mRNA expression that was limited to Fc-TWEAK-treated healthy littermates (Fig. 7x). These results suggest that the impact of Fc-TWEAK on molecular markers associated with muscle health may be dependent on age, disease severity and/or genetic strain. Despite the lack of impact of Fc-TWEAK on muscle atrophy and health markers, quantification of myofiber area in TA muscles shows a significant increase in muscle size in Fc-TWEAK-treated *Smn*$^{2B/-}$ mice compared to untreated SMA animals (Fig. 7y-z).

While MuRF-1 and atrogin-1 are well described atrophy markers [57], whose expression has previously been well characterized in skeletal muscle of *Smn*$^{-/-}$*;SMN2* and *Smn*$^{2B/-}$ mice at various time-points during disease progression [61], there is also evidence that they can be induced by the Tweak/Fn14 signaling

cascade [24]. We therefore investigated their levels in quadriceps and triceps of P7 *Fn14*$^{-/-}$ mice (Supplementary Fig. 5) and find that whilst *atrogin-1* levels are unchanged compared to WT animals (Supplementary Fig. 5a), *MuRF-1* levels are significantly downregulated in both muscles of *Fn14*$^{-/-}$ mice, consistent with the previously reported positive correlation between Tweak/Fn14 activity and MuRF-1 expression (Supplementary Fig. 5b) [24]. These results suggest that the reduced levels of *MuRF-1* observed in skeletal muscle of Fc-TWEAK-treated SMA mice are most likely linked to improved muscle health. Furthermore, the differential effect of Fc-TWEAK on the expression of *MuRF-1* and *atrogin-1* in *Smn*$^{-/-}$*;SMN2* and *Smn*$^{2B/-}$ is most probably due to the previously reported distinct regulatory processes that contribute to muscle atrophy in both models [61].

Taken together, our results suggests that promoting Tweak activity in SMA mice has the potential to improve weight, survival, and muscle pathology, suggesting that restoring the Tweak and Fn14 signaling in SMA muscle may lead to sustainable therapeutic benefits.

DISCUSSION

Motor neuron death and muscle pathology bi-directionally impact on each other in SMA. Indeed, while loss of motor neurons significantly contributes to muscle atrophy, there is also evidence for muscle-intrinsic abnormalities in SMA skeletal muscle, which could be directly or indirectly caused by SMN deficiency [5,6,62–64]. In this study, we attempted to address the underlying mechanisms of muscle-intrinsic abnormalities leading to muscle pathology in SMA by investigating the role of TWEAK and Fn14 in muscle atrophy in SMA. To the best of our knowledge, this is the first study to evaluate the TWEAK and Fn14 pathway in SMA and in early stages of muscle development.

Notably, we showed decreased expression of *Tweak* and *Fn14* in skeletal muscle of two distinct SMA mouse models during disease progression, which is contrary to previous reports of increased TWEAK/Fn14 activity in experimental models of atrophy in adult muscle [52,65,66], suggesting that TWEAK and Fn14 may have distinct roles in skeletal muscle during development and adulthood. Indeed, *Tweak* mRNA expression is significantly lower in skeletal muscle of 30-day-old WT mice compared to 90-day-old animals, suggesting an age-dependent regulation [16]. Moreover, we observed that the dysregulation of TWEAK, Fn14 and the previously proposed metabolic effectors in skeletal muscle of pre-weaned mice appears to be influenced by intrinsic muscle impairments and not denervation, which is in contrast to what has been previously reported in experimental models of adult muscle denervation [15,16], further suggesting distinct developmental roles for Tweak and Fn14 in skeletal muscle. Given that muscles from younger mice are more resistant to surgically-induced denervation than in older mice [67], TWEAK and Fn14 may contribute to this age-dependent differential vulnerability of muscle to pathological insults. Thus, the role of TWEAK/Fn14 signaling in muscle pathology may be more nuanced and be influenced by a combination of factors such as absolute levels, downstream signaling cascades activated (e.g. canonical vs non-canonical NF-κB signaling pathways), developmental stage of the muscle, state of muscle atrophy (e.g. chronic vs acute) and primary origin of muscle pathology (e.g. denervation vs intrinsic insult) [11,12].

598 Another key observation from our study is a potential interaction and/or overlap between Tweak, Fn14 and
599 Smn and their downstream signaling cascades in muscle. It has previously been demonstrated that once
600 Tweak binds to Fn14, the complex will activate several NF-κB molecular effectors, including TRAF6 and
601 IKK [68]. Interestingly, SMN has been reported to prevent the activation of TRAF6 and IKK, thereby
602 negatively regulating the muscle atrophy-inducing canonical NF-κB pathway [69]. These studies thus
603 suggest converging roles for TWEAK, Fn14 and Smn in muscle, which is further supported by our findings.
604 Indeed, we found that independent *Tweak*, *Fn14* and *Smn* depletion had an impact on each other's expression
605 in differentiated C2C12 cells and murine muscle. Furthermore, there was an overlap of dysregulated
606 myogenesis, myopathy and glucose metabolism genes in SMA, $Fn14^{-/-}$ and $Tweak^{-/-}$ mice. Of note, the
607 aberrantly regulated genes in young $Tweak^{-/-}$ and $Fn14^{-/-}$ mice did not perfectly overlap, supporting previous
608 reports of Tweak-independent roles of Fn14 during myogenesis [70]. Thus, these results suggest that aberrant
609 expression of TWEAK and Fn14 in SMA muscle may be a consequence of combined events resulting from
610 muscle atrophy and reduced SMN expression. However, Smn depletion most likely needs to reach
611 pathological levels as we did not observe obvious changes in the Tweak/Fn14 signaling pathway in skeletal
612 muscle of non-SMA hypomorphic $Smn^{2B/2B}$ and $Smn^{+/-}$ mice. Performing genome-wide RNA sequencing
613 studies could also help elucidate the extent of shared genes and pathways regulated by TWEAK, Fn14 and
614 SMN. Indeed, while we have focused on a subset of previously reported and proposed metabolic effectors
615 and the NF-κB pathways, other canonical pathways such as MAPK signaling, known to have functional
616 interactions with Tweak, Fn14 and Smn, may also display converging roles in muscle health [71,72].
617
618 In addition, our results in developing mice do support the previously reported negative regulation of the
619 metabolic factors Pgc-1α, Mef2d, Glut-4, Klf15, and HKII in adult muscle [18]. Further analyses of a subset
620 of specific glucose metabolism genes showed that about 20% of these genes were dysregulated in the same
621 direction in $Fn14^{-/-}$, $Tweak^{-/-}$ and SMA mice. Our KEGG analysis of these shared dysregulated metabolic
622 genes further support the potential relationships and roles of TWEAK, Fn14 and SMN involved in the

623 regulation of glucose metabolism. Indeed, the AMPK signaling pathway, found to be aberrantly regulated
624 in *Fn14*$^{-/-}$, *Tweak*$^{-/-}$ and SMA, is as a master regulator of skeletal muscle function and metabolism [73].
625 Interestingly, a previous study in *SMNΔ7* SMA mice further showed that chronic treatment with the AMPK
626 agonist AICAR prevented skeletal muscle pathology [74]. In addition, AMPK directly phosphorylates PGC-
627 1α [75], which is also dysregulated in *Smn*-, *Tweak*- and *Fn14*-depleted models [66,76]. We also found that
628 glycolysis and pyruvate metabolic pathways, which culminate in the generation of ATP, are also
629 dysregulated in SMA, *Fn14*$^{-/-}$ and *Tweak*$^{-/-}$ mice. Interestingly, siRNA-mediated *Smn* knockdown in NSC-
630 34 cells showed a significant decrease in ATP production [77]. ATP was also decreased in *Smn*$^{-/-}$;*SMN2* mice
631 and in Smn morphant zebrafish [78]. These results could explain mitochondrial dysfunction in SMA patients
632 [5]. Thus, our study strengthens the notion of metabolic dysfunctions contributing to SMA muscle pathology
633 and suggests a potential mechanistic link with the TWEAK/Fn14 pathway.
634
635 However, it is important to note that although our findings support the idea that the aberrant expression of
636 *Pgc-1α*, *Mef2d*, *Glut-4*, *Klf15* and *HKII* is due to the dysregulated expression of *Tweak* and *Fn14* in SMA
637 muscle, further mechanistic insights are required to fully understand the extent of the transcriptional
638 regulation of these key metabolic effectors by TWEAK/Fn14 signaling in developing post-natal muscle.
639 Indeed, their differential dysregulations in *Smn*$^{-/-}$;*SMN2*, *Smn*$^{2B/-}$, *Tweak*$^{-/-}$ and *Fn14*$^{-/-}$ muscle as well as the
640 varying impact that Fc-Tweak injections had on their expression levels suggest that additional regulatory
641 mechanisms may be contributing to our observations.
642
643 Our findings also confirm that not all skeletal muscles are equally affected in SMA. Indeed, we observed
644 that the SMA skeletal muscle atrophy marker *parvalbumin* was significantly decreased from an earlier
645 timepoint in the vulnerable triceps and gastrocnemius muscles than in the more resistant TA and quadriceps
646 muscles. Notably, we also found that 20% more myogenesis- and myopathy-related genes were dysregulated
647 in the more vulnerable triceps muscles of *Smn*$^{-/-}$;*SMN2* mice compared to the resistant quadriceps muscles.

Conversely, the number of glucose metabolism genes dysregulated in SMA triceps and quadriceps muscles was not significantly different. Previous studies have reported that muscle vulnerability is more closely associated with NMJ denervation than with location or fibre type composition [35]. Our results further suggest that denervation events in vulnerable SMA muscles have a more prominent effect on myogenesis and myopathy than on glucose metabolism.

Finally, modulating Tweak activity via Fc-TWEAK in two SMA mouse models led to interesting observations. Firstly, Fc-TWEAK administration specifically increased lifespan in the milder *Smn*$^{2B/-}$ mouse model, while it did not impact disease progression in the severe *Smn*$^{-/-}$;*SMN2* mice. This is consistent with previous studies, including ours, demonstrating that the *Smn*$^{2B/-}$ mice are more responsive to non-SMN interventions, perhaps due to their longer asymptomatic, and therefore adaptable, period [41,58–60,79]. At a molecular level, we found that Fc-TWEAK differentially impacted the expression of the *Tweak*, *Fn14* and the previously proposed metabolic effectors in SMA mice and healthy littermates in a time-dependent manner, perhaps reflecting disease state-dependent regulatory mechanisms of the pathway. Importantly, the expression of *Mef2d*, *HKII* and *Klf15* was significantly downregulated in Fc-TWEAK-treated SMA mice, supporting an increased activity of Tweak in the mice and a subsequent restoration towards normal levels of aberrantly expressed proposed Tweak/Fn14 effectors. As mentioned above, the timing between Fc-TWEAK administration and tissue collection may have limited our analysis of the effect of Fc-TWEAK on the Tweak/Fn14 signaling cascade. Furthermore, our focus on the specific subset of previously reported proposed metabolic effectors (*Pgc-1α*, *Mef2d*, *Glut-4*, *Klf15* and *HKII*) probably also resulted in us not having a complete picture of the molecular impacts of Fc-TWEAK. Indeed, Fc-TWEAK may have affected the expression of the shared aberrantly expressed genes identified with the myogenesis, myopathy and glucose metabolism PCR arrays such as *Pax7* and *Titin* as well as the above mentioned pathways (e.g. MAPK and AMPK). Nevertheless, administration of Fc-Tweak did improve muscle pathology in SMA mice as demonstrated by the partial restoration of molecular markers of muscle health and myofiber size. These

results support a role for the TWEAK/Fn14 pathway in maintaining skeletal muscle health and homeostasis [12]. However, it is important to note that the TWEAK/Fn14 pathway is involved in many other tissues and pathologies such as tumor development and metastasis, heart-related diseases [80], kidney injury, cerebral ischemia [81,82] and autoimmune diseases [83,84], which could have influenced the overall impact of systemically administered Fc-TWEAK on muscle health and disease progression in SMA mice.

CONCLUSION

In summary, our results suggest a potential role and contribution of the TWEAK/Fn14 pathway to myopathy and glucose metabolism perturbations in SMA muscle. Furthermore, our study, combined with previous work in adult models [11,12], proposes that dysregulation of the TWEAK/Fn14 signaling in muscle appears to be dependent on the origin of the muscle pathology (e.g. denervation vs intrinsic) and developmental stage of skeletal muscle (e.g. newborn, juvenile, adult, aged), further highlighting the differential and conflicting activities of the pathway. Future investigations should therefore be aimed at both furthering our understanding of the relevance of the Tweak/Fn14 pathway in SMA muscle and defining its role in general in maintaining muscle homeostasis throughout the life course.

LIST OF ABBREVIATIONS

ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
cDNA	complementary deoxyribonucleic acid
DEG	differently expressed genes
DMEM	Dulbecco's Modified Eagle's Media
FBS	fetal bovine serum
FDR	false discovery rate
GO	gene ontology
H&E	hematoxylin-and-eosin
KEGG	Kyoto Encyclopedia of Genes and Genomes
mRNA	messenger RNA
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMJ	neuromuscular junctions
P	postnatal day
p	probability value
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
qPCR	quantitative polymerase chain reaction
RIPA	radioimmunoprecipitation
RNA	ribonucleic acid
RNAi	RNA interference
RT-qPCR	reverse transcriptase-quantitative PCR
SEM	standard error of the mean

siRNA	small interfering RNA
SMA	spinal muscular atrophy
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TA	tibialis anterior
WT	wild type

DECLARATIONS

Ethics approval and consent to participate

Experimental procedures with live animals were authorized and approved by the University of Oxford ethics committee and UK Home Office (current project license PDFEDC6F0, previous project license 30/2907) in accordance with the Animals (Scientific Procedures) Act 1986, the Keele University Animal Welfare Ethical Review Body and UK Home Office (Project Licence P99AB3B95) in accordance with the Animals (Scientific Procedures) Act 1986, the University of Ottawa Animal Care Committee according to procedures authorized by the Canadian Council on Animal Care and the German Animal Welfare law and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, reference numbers 15/1774 and 19/3309).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article or in the supplementary information.

Competing interests

The authors declare they have no competing interests.

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Authors' contributions

Conceptualization: M.B.; Methodology: K.E.M., E.R.S., M.B.; Validation: K.E.M., M.B.; Formal analysis: K.E.M., E.R.S., E.M., E.M., S.K., M.B.; Investigations: K.E.M., E.R.S., E.M., E.M., D.A., B.E., S.K., I.T., I.B., G.H., N.A., M.B.; Writing - original draft preparation: K.E.M, M.B.; Writing – review and editing: K.E.M., E.R.S., E.M., E.M., D.A., B.E., S.K., I.T., I.B., G.H., N.A., P.C., K.E.D., R.K., M.J.A.W., M.B.; Visualization: K.E.M., E.R.S., E.M., M.B.; Supervision: P.C., K.E.D., R.K., M.J.A.W., M.B.; Project administration: M.B.; Funding acquisition: R.K., M.J.A.W., M.B.

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FIGURE LEGENDS

Figure 1. Aberrant expression of Tweak and Fn14 in skeletal muscle of *Smn$^{-/-}$;SMN2* SMA mice. a-g. qPCR analysis of *parvalbumin* (**a**)**,** *Tweak* (**b**), *Fn14* (**c**), *Pgc-1α* (**d**), *Mef2d* (**e**), *Glut-4* (**f**) and *HKII* (**g**) in triceps, gastrocnemius, TA and quadriceps muscles from post-natal day (P) 0 (birth), P2 (pre-symptomatic), P5 (early-symptomatic), P7 (late symptomatic) and P19 (end-stage) *Smn$^{-/-}$;SMN2* and wild-type (WT) mice. Normalized relative expressions are compared to WT P0. Data are mean ± SEM, n = 3-4 animals per experimental group, two-way ANOVA, Sidak's multiple comparison test between genotypes, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. **h-i**. Quantification of NF-κB p50/actin protein levels in the TA of pre-symptomatic (P2) (**h**) and late-symptomatic (P7) (**i**) *Smn$^{-/-}$;SMN2* mice and age-matched WT animals. Images are representative immunoblots. Data are mean ± SEM, n = 3-4 animals per experimental group**,** unpaired *t* test, ns = not significant (**h**), $p = 0.0215$ (**i**). **j.** Quantification of NF-κB p50/actin and p105/actin protein levels in the quadriceps (quad) of late-symptomatic (P7) *Smn$^{-/-}$;SMN2* mice and age-matched WT animals. Images are representative immunoblots. Data are mean ± SEM, n = 3-4 animals per experimental group**,** two-way ANOVA, uncorrected Fisher's LSD, **** $p < 0.0001$, ns = not significant.**k.** qPCR analysis *NF-κB inducing kinase* (*NIK*) in TA muscle of late-symptomatic P7 *Smn$^{-/-}$;SMN2* and age-matched WT animals. Data are mean ± SEM, n = 3-4 animals per experimental group**,** unpaired *t* test, $p = 0.0094$.

Figure 2. Aberrant expression of Tweak and Fn14 in skeletal muscle of *Smn$^{2B/-}$* SMA mice. a-g. qPCR analysis of *parvalbumin* (**a**)**,** *Tweak* (**b**), *Fn14* (**c**), *Pgc-1α* (**d**), *Mef2d* (**e**), *Glut-4* (**f**) and *HKII* (**g**) in TA muscles from P0 (birth), P2 (pre-symptomatic), P4 (pre-symptomatic), P11 (early symptomatic) and P19 (end-stage) *Smn$^{2B/-}$* and WT mice. Normalized relative expressions are compared to WT P0. Data are mean ± SEM, n = 3-4 animals per experimental group, two-way ANOVA, Sidak's multiple comparison test between genotypes, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. **h**. Quantification of Tweak protein levels normalized to total protein in the triceps of late-symptomatic (P18) *Smn$^{2B/-}$* mice and age-

matched WT animals. Images are representative immunoblots. Data are mean ± SEM, n = 6-7 animals per experimental group**,** unpaired *t* test, $p = 0.014$. **i**. Quantification of NF-κB1 p50 and p105 protein levels normalized to total protein in the triceps of late-symptomatic (P18) $Smn^{2B/-}$ mice and age-matched WT animals. Images are representative immunoblots. Data are mean ± SEM, n = 6-7 animals per experimental group**,** unpaired *t* test, ns = not significant (p50), $p = 0.0354$ (p105). **j**. Quantification of NF-κB2 p52 and p100 protein levels normalized to total protein in the triceps of late-symptomatic (P18) $Smn^{2B/-}$ mice and age-matched WT animals. Images are representative immunoblots. Data are mean ± SEM, n = 3-4 animals per experimental group**,** unpaired *t* test, ns = not significant (p52), $p = 0.0532$ (p100).

Figure 3. Tweak and Fn14 are not dysregulated in denervated (nerve crush) muscles of pre-weaned mice. A sciatic nerve crush was performed on post-natal day (P) 7 WT FVB/N mice and both ipsilateral (nerve crush) and contralateral (control) TA muscles were harvested at P14. **a.** Representative images of hematoxylin and eosin-stained cross-sections of control and nerve crush TA muscles. Scale bars = 100 µm. **b.** Myofiber area in control and nerve crush TA muscles. Data are mean ± SEM, n = 3-6 animals per experimental group**,** unpaired *t* test, $p = 0.0020$. **c.** Myofiber size distribution in control and nerve crush TA muscles. **d.** qPCR analysis of *parvalbumin***,** *Tweak*, *Fn14*, *Pgc-1α*, *Mef2d*, *Glut-4*, *HKII*, *Klf15* and *Smn* in control and nerve crush TA muscles. Normalized relative expressions for each gene are compared to control muscle. Data are mean ± SEM, n = 4-6 animals per experimental group, two-way ANOVA, uncorrected Fisher's LSD, ns = not significant.

Figure 4. Tweak and Fn14 are dysregulated in cardiotoxin-induced muscle necrosis in pre-weaned mice. Cardiotoxin was injected in the left TA muscle of post-natal day (P) 10. The right TA muscle was injected with equal volumes of 0.9% saline. TA muscles were harvested 6 days later. **a.** Representative images of hematoxylin and eosin-stained cross-sections of saline- and cardiotoxin-injected TA muscles. Scale bars = 100 µm. **b.** Percentage of muscle fibers with centrally-located nuclei in saline- and cardiotoxin-

injected TA muscles. Data are mean ± SEM, n = 3 animals per experimental group**,** unpaired *t* test, p = 0.0020. **c.** qPCR analysis of *parvalbumin***,** *Tweak, Fn14, Pgc-1α, Mef2d, Glut-4, HKII, Klf15* and *Smn* in saline- and cardiotoxin-injected TA muscles. Normalized relative expressions for each gene are compared to saline-treated muscle. Data are mean ± SEM, n = 3 animals per experimental group, two-way ANOVA, uncorrected Fisher's LSD, ns = not significant, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 5. *Smn*, *Tweak* and *Fn14* depletion impact each other's expression. a-b. qPCR analysis of *parvalbumin***,** *Tweak, Fn14, Pgc-1α, Mef2d, Glut-4, HKII, Klf15* and *Smn* in triceps muscle from post-natal day (P) 7 *Tweak*$^{-/-}$ (**a**) and *Fn14*$^{-/-}$ (**b**) mice. Normalized relative expressions for each gene are compared to WT. Data are mean ± SEM, n = 4 animals per experimental group, two-way ANOVA, uncorrected Fisher's LSD, ns = not significant, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. **c-j**. qPCR analysis of *Smn* (**c**), *Tweak* (**d**), *Fn14* (**e**), *Pgc-1α* (**f**), *Mef2d* (**g**), *Glut-4* (**h**), *HKII* (**i**) and *Klf15* (**j**) in siRNA-mediated *Tweak*-, *Fn14*- and *Smn*-depleted and control proliferating (Day 0) and differentiated (Day 7) C2C12 cells. Normalized relative expressions for Day 1 experimental groups are compared to Day 1 untreated group and normalized relative expressions for Day 7 experimental groups are compared to Day 7 untreated group. Data are mean ± SEM, n = 3 per experimental group, two-way ANOVA, Dunnett's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. **k.** Proposed model of the relationship between Smn and the Tweak/Fn14 signaling pathway. Red lines represent inhibition and blue lines represent activation.

Figure 6. Overlap between dysregulated genes involved in myopathy, myogenesis and glucose metabolism in skeletal muscle of *Smn*$^{-/-}$*;SMN2*, *Fn14*$^{-/-}$ and *Tweak*$^{-/-}$ mice. a. Venn diagram showing overlap of genes involved in myopathy and myogenesis that are significantly dysregulated in the same direction (either up or downregulated, $p < 0.05$) in triceps and quadriceps muscle from post-natal day (P) 7 compared to *Smn*$^{-/-}$*;SMN2, Fn14*$^{-/-}$ and *Tweak*$^{-/-}$ mice to age- and genetic strain-matched wild type (WT) mice. **b.** Network and enrichment analysis of the overlap of significantly dysregulated myopathy and

myogenesis genes in triceps and/or quadriceps of P7 *Smn*$^{-/-}$*;SMN2*, *Fn14*$^{-/-}$ and *Tweak*$^{-/-}$ mice using STRING software. Smn (Smn1), TWEAK (Tnfsf12) and Fn14 (Tnfrsf12a) are included in the analysis. Corresponding protein nodes in the network are highlighted in color. The connection color and shape between proteins represent protein-protein associations (Action types) and if the association is positive, negative or unspecified (Action effects). **c.** Venn diagram showing overlap of genes involved in glucose metabolism that are significantly dysregulated in the same direction (either up or downregulated, $p < 0.05$) in triceps and quadriceps muscle from P7 compared to *Smn*$^{-/-}$*;SMN2*, *Fn14*$^{-/-}$ and *Tweak*$^{-/-}$ mice to age- and genetic strain-matched WT mice. **d.** Network and enrichment analysis of the overlap of significantly dysregulated myopathy and myogenesis genes in triceps and/or quadriceps of P7 *Smn*$^{-/-}$*;SMN2*, *Fn14*$^{-/-}$ and *Tweak*$^{-/-}$ mice using STRING software. Smn (Smn1), TWEAK (Tnfsf12), Fn14 (Tnfrsf12a), HKII (Hk2), Glut4 (Slc2a4), Pgc-1α (Ppargc1a), Klf15 and Mef2d are included in the analysis. Corresponding protein KEGG pathways with the six lowest FDRs highlighted in color (see Table 3). The connection color and shape between proteins represent protein-protein associations (Action types) and if the association is positive, negative or unspecified (Action effects).

Figure 7. Increasing Tweak activity via Fc-TWEAK improves disease phenotypes in two SMA mouse models. a. Daily weights of untreated *Smn*$^{-/-}$*;SMN2* SMA mice and *Smn*$^{-/-}$*;SMN2* mice that received daily subcutaneous injections (starting at P0) of Fc-TWEAK or IgG control (15.8 µg). Data are mean ± SEM, n = 7-10 animals per experimental group, two-way ANOVA, Sidak's multiple comparison test. **b.** Survival curves of untreated *Smn*$^{-/-}$*;SMN2* SMA mice and *Smn*$^{-/-}$*;SMN2* that received daily subcutaneous injections of Fc-TWEAK or IgG control (15.8 µg). Data are represented as Kaplan-Meier survival curves, n = 7-10 animals per experimental group, Log-rank (Mantel-Cox). **c-i.** qPCR analysis of *Tweak* (**c**), *Fn14* (**d**), *Pgc-1α* (**e**), *Mef2d* (**f**), *Glut-4* (**g**), *HKII* (**h**) and *Klf15* (**i**) in triceps of post-natal day (P) 7 untreated and Fc-TWEAK-treated (15.8 µg) *Smn*$^{-/-}$*;SMN2* SMA and *Smn*$^{+/-}$*;SMN2* health littermates. Normalized relative expressions for Fc-TWEAK-treated *Smn*$^{+/-}$*;SMN2* mice are compared to untreated *Smn*$^{+/-}$*;SMN2* mice and

normalized relative expressions for Fc-TWEAK-treated *Smn*$^{-/-}$*;SMN2* mice are compared to untreated *Smn*$^{-/-}$*;SMN2* mice. Data are mean ± SEM, n = 3-4 animals per experimental group, two-way ANOVA, uncorrected Fisher's LSD between genotypes, * $p < 0.05$, *** $p < 0.001$. **j**. Representative images of laminin-stained cross-sections of TA muscles from P7 untreated and Fc-TWEAK-treated (15.8 µg) *Smn*$^{-/-}$*;SMN2* SMA and *Smn*$^{+/-}$*;SMN2* health littermates (Scale bars = 100 µm) and quantification of myofiber area. Data are mean ± SEM, n = 3-4 animals per experimental group (>550 myofibers per experimental group), one-way ANOVA, Tukey's multiple comparison test, * $p < 0.05$, **** $p < 0.0001$. **k**. Relative frequency distribution of myofiber size in TA muscles of P7 untreated and Fc-TWEAK-treated (15.8 µg) *Smn*$^{-/-}$*;SMN2* SMA and *Smn*$^{+/-}$*;SMN2* health littermates. **l-p**. qPCR analysis of *parvalbumin* (**l**), *MuRF-1* (**m**), *atrogin-1* (**n**), *Myod1* (**o**), and *myogenin* (**p**) in triceps of P7 untreated and Fc-TWEAK-treated (15.8 µg) *Smn*$^{-/-}$*;SMN2* SMA and *Smn*$^{+/-}$*;SMN2* health littermates. Normalized relative expressions for Fc-TWEAK-treated *Smn*$^{+/-}$*;SMN2* mice are compared to untreated *Smn*$^{+/-}$*;SMN2* mice and normalized relative expressions for Fc-TWEAK-treated *Smn*$^{-/-}$*;SMN2* mice are compared to untreated *Smn*$^{-/-}$*;SMN2* mice. Data are mean ± SEM, n = 3-4 animals per experimental group, two-way ANOVA, uncorrected Fisher's LSD between genotypes, * $p < 0.05$, ** $p < 0.01$. **q**. Daily weights of untreated *Smn*$^{2B/-}$ SMA mice and *Smn*$^{2B/-}$ mice that received subcutaneous injections of Fc-TWEAK or IgG control (15.8 µg) every 4 days (starting at P0). Data are mean ± SEM, n = 9-12 animals per experimental group, two-way ANOVA, Sidak's multiple comparison test. **r**. Survival curves of untreated *Smn*$^{2B/-}$ SMA mice and *Smn*$^{2B/-}$ mice that received subcutaneous injections of Fc-TWEAK or IgG control (15.8 µg) every 4 days (starting at P0). Data are Kaplan-Meier survival curves, n = 9-12 animals per experimental group, Log-rank (Mantel-Cox), $p = 0.0162$. **s-x**. qPCR analysis of *Glut-4* (**s**), *parvalbumin* (**t**), *MuRF-1* (**u**), *atrogin-1* (**v**), *Myod1* (**w**) and *myogenin* (**x**) in P15 untreated and Fc-TWEAK-treated (15.8 µg) *Smn*$^{2B/+}$ and *Smn*$^{2B/-}$ mice (every 4 days starting at P0). Normalized relative expressions for Fc-TWEAK-treated *Smn*$^{2B/+}$ mice are compared to untreated *Smn*$^{2B/+}$ mice and normalized relative expressions for Fc-TWEAK-treated *Smn*$^{2B/-}$ mice are compared to untreated *Smn*$^{2B/-}$ mice. Data are mean ± SEM, n = 3-4 animals per experimental group, two-way ANOVA,

uncorrected Fisher's LSD between genotypes, * $p < 0.05$, *** $p < 0.001$, ns = not significant. **y**. Representative images of laminin-stained cross-sections of TA muscles from P16 untreated and Fc-TWEAK-treated (15.8 µg every 4 days starting at P0) *Smn*$^{2B/+}$ and *Smn*$^{2B/-}$ mice (Scale bars = 50 µm) and quantification of myofiber area. Data are mean ± SEM, n = 3-7 animals per experimental group (>400 myofibers per experimental group), one-way ANOVA, Tukey's multiple comparison test, * $p < 0.05$, **** $p < 0.0001$. **z**. Relative frequency distribution of myofiber size in TA muscles of P16 untreated and Fc-TWEAK-treated (15.8 µg every 4 days starting at P0) *Smn*$^{2B/+}$ and *Smn*$^{2B/-}$ mice.

TABLES

Table 1. Myogenesis and myopathy genes significantly dysregulated in the same direction in triceps and quadriceps of P7 *Smn*$^{-/-}$;*SMN2*, *Fn14*$^{-/-}$ and *Tweak*$^{-/-}$ mice when compared to P7 WT mice.

Table 2. Glucose metabolism genes significantly dysregulated in the same direction in triceps and quadriceps of P7 *Smn*$^{-/-}$;*SMN2*, *Fn14*$^{-/-}$ and *Tweak*$^{-/-}$ mice when compared to P7 WT mice.

Table 3. KEGG pathways generated from glucose metabolism genes that were are significantly dysregulated in the same direction in triceps and quadriceps of P7 *Smn*$^{-/-}$;*SMN2*, *Fn14*$^{-/-}$ and *Tweak*$^{-/-}$ mice when compared to P7 WT mice.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. No overt dysregulation of Tweak and Fn14 in skeletal muscle of non-SMA hypomorphic Smn-depleted mice. qPCR analysis of *Tweak* (a), *Fn14* (b), *Pgc-1α* (c), *Mef2d* (d), *Glut*-4 (e), *HKII* (f) and *Klf15* (g) in triceps from post-natal day (P) 7 wild-type (WT), $Smn^{2B/2B}$ and $Smn^{+/-}$ mice. Normalized relative expressions are compared to WT. Data are mean ± SEM, n = 4-5 animals per experimental group, one-way ANOVA, Tukey's multiple comparison test, * $p < 0.05$, ns = not significant.

Supplementary Figure 2. Tweak and Fn14 are not dysregulated in denervated (nerve cut) muscles of pre-weaned mice. A sciatic nerve cut was performed on post-natal day (P) 7 WT FVB/N mice and both ipsilateral (nerve cut) and contralateral (control) TA muscles were harvested at P14. qPCR analysis of *Tweak*, *Fn14*, *Pgc-1α*, *Mef2d*, *Glut-4*, *HKII* and *Klf15* in control and nerve cut TA muscles. Normalized relative expressions for each gene are compared to control muscle. Data are mean ± SEM, n = 7-11 animals per experimental group, two-way ANOVA, uncorrected Fisher's LSD, ns = not significant.

Supplementary Figure 3. Effect of varying Fc-TWEAK doses on disease progression in $Smn^{-/-}$;*SMN2* SMA mice. $Smn^{-/-}$;*SMN2* mice received daily subcutaneous injections of increasing doses of Fc-TWEAK (7.9, 15., 23.7 and 31.6 μg), starting at birth. **a**. Daily weights of untreated $Smn^{-/-}$;*SMN2* SMA mice and $Smn^{-/-}$;*SMN2* mice that received daily subcutaneous injections (starting at P0) of Fc-TWEAK (7.9, 15.8, 23.7 and 31.6 μg). Data are mean ± SEM, n = 5-10 animals per experimental group, two-way ANOVA, Sidak's multiple comparison test. **b**. Survival curves of untreated $Smn^{-/-}$;*SMN2* SMA mice and $Smn^{-/-}$;*SMN2* mice that received daily subcutaneous injections (starting at P0) of Fc-TWEAK (7.9, 15.8, 23.7 and 31.6 μg). Data are presented as Kaplan-Meier survival curves, n = 5-10 animals per experimental group, Log-rank (Mantel-Cox).

Supplementary Figure 4. Differential effect of Fc-TWEAK in skeletal muscle of wild type (WT) and *Smn$^{2B/-}$* mice. WT and *Smn$^{2B/-}$* SMA mice and received subcutaneous injections of Fc-TWEAK (15.8 µg) every 4 days (from post-natal day (P) 0 to P16) and skeletal muscles were harvested 3 hours post-injections. a-g. qPCR analysis of *Fn14* (**a**), *Tweak* (**b**), *Pgc-1α* (**c**), *Mef2d* (**d**), *Glut-4* (**e**), *HKII* (**f**) and *Klf15* (**g**) in triceps of untreated and Fc-TWEAK-treated *Smn$^{2B/-}$* mice. Normalized relative expressions for Fc-TWEAK-treated WT mice are compared to untreated WT mice and normalized relative expressions for Fc-TWEAK-treated *Smn$^{2B/-}$* mice are compared to untreated *Smn$^{2B/-}$* mice. Data are mean ± SEM, n = 3-5 animals per experimental group, two-way ANOVA, uncorrected Fisher's LSD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant. **h**. Quantification of NF-κB2 p100 protein levels normalized to total protein in the quadriceps of late-symptomatic (P18) *Smn$^{2B/-}$* mice and age-matched WT animals. Images are representative immunoblots. Data are mean ± SEM, n = 3-5 animals per experimental group**,** unpaired *t* test, $p = 0.0005$ (WT), $p = 0.0494$.

Supplementary Figure 5. Decreased *MuRF-1* expression in skeletal muscle of P7 *Fn14$^{-/-}$* mice. qPCR analysis of *Atrogin-1* (**a**) and *MuRF-1* (**b**) in quadriceps and triceps from post-natal day (P) 7 wild type (WT) and *Fn14$^{-/-}$* mice. Normalized relative expressions are compared to WT. Data are mean ± SEM, n = 4 animals per experimental group, unpaired *t* test, $p = 0.0164$ (*MuRF-1* quadriceps), $p = 0.0283$ (*MuRF-1* triceps), ns = not significant.

SUPPLEMENTARY TABLES

Supplementary Table 1. Mouse primers used for quantitative real-time PCR.

SUPPLEMENTARY FILES

Supplementary File 1. Myopathy and myogenesis gene expression changes in triceps and quadriceps of post-natal day 7 *Smn$^{-/-}$;SMN2* (SMA), *Tweak$^{-/-}$* (Tweak KO) and *Fn14$^{-/-}$*; (Fn14 KO) compared to age- and genetic strain-matched wild type animals.

Supplementary File 2. Glucose metabolism gene expression changes in triceps and quadriceps of post-natal day 7 *Smn$^{-/-}$;SMN2* (SMA), *Tweak$^{-/-}$* (Tweak KO) and *Fn14$^{-/-}$*; (Fn14 KO) compared to age- and genetic strain-matched wild type animals.