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A Comparison of Three Electrophysiological Methods for the Assessment of Disease Status in a Mild Spinal Muscular Atrophy Mouse Model

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Abstract

Objectives: There is a need for better, noninvasive quantitative biomarkers for assessing the rate of progression and possible response to therapy in spinal muscular atrophy (SMA). In this study, we compared three electrophysiological measures: compound muscle action potential (CMAP) amplitude, motor unit number estimate (MUNE), and electrical impedance myography (EIM) 50 kHz phase values in a mild mouse model of spinal muscular atrophy, the *Smn1^{c/c}* mouse.

Methods: *Smn1^{c/c}* mice (N=11) and wild type (WT) animals ($-/-$, N=13) were measured on average triweekly until approximately 1 year of age. Measurements included CMAP, EIM, and MUNE of the gastrocnemius muscle as well as weight and front paw grip strength. At the time of sacrifice at one year, additional analyses were performed on the animals including serum survival motor neuron (SMN) protein levels and muscle fiber size.

Results: Both EIM 50 kHz phase and CMAP showed strong differences between WT and SMA animals (repeated measures 2-way ANOVA, $P < 0.0001$ for both) whereas MUNE did not. Both body weight and EIM showed differences in the trajectory over time ($p < 0.001$ and $p = 0.005$, respectively). At the time of sacrifice at one year, EIM values correlated to motor neuron counts in the spinal cord and SMN levels across both groups of animals ($r = 0.41$, $p = 0.047$ and $r = 0.57$, $p = 0.003$, respectively), while CMAP did not. Motor neuron number in *Smn1^{c/c}* mice was not significantly reduced compared to WT animals.

Conclusions: EIM appears sensitive to muscle status in this mild animal model of SMA. The lack of a reduction in MUNE or motor neuron number but reduced EIM and CMAP values support that much of the pathology in these animals is distal to the cell body, likely at the neuromuscular junction or the muscle itself.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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Competing Interests: Dr. Rutkove has equity in, and serves a consultant and scientific advisor to, Skulpt, Inc., a company that designs impedance devices for clinical and research use; he is also a member of the company's Board of Directors. The company also has an option to license patented impedance technology of which Dr. Rutkove is named as an inventor. In addition, Phillip Zaworski, is employed by PharmOptima, Inc. and is named on a patent application (Methods for the detecting survival motor neuron (SMN) protein in whole blood or cerebral spinal fluid, U.S. Provisional Application No 61/834,325). These facts do not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

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Introduction

A variety of mouse models of spinal muscular atrophy (SMA) have been developed over the past decade [1]. These include animals with relatively severe phenotypes, such as the survival motor neuron negative (*Smn^{-/-}*), *Smn2^{+/+}*, and SMNΔ7 models [2–4], all of which die within the first 3 weeks of life, to relatively long lived models, including the *Smn1^{c/c}* mouse, which develops only subtle motor deficits [5]. The impetus underlying the development of these various models is based on an effort to recapitulate the marked varying human disease severities, with the SMNΔ7 model mimicking Type 1 SMA and *Smn1^{c/c}* mimicking SMA Type 3. While each of these disease models has its unique

features, nearly all of the longer-lived models develop some element of digital, ear, and tail necrosis [5].

A major purpose of these models is to evaluate potential therapies. Beyond measuring survival, which is effective in the study of more severe models [6,7], biomarkers are needed to evaluate more subtle therapeutic effects, such as disease stabilization or slowing of progression. A variety of such biomarkers have been explored in both animals and humans, including serological biomarkers measures such as SMN expression [8], functional measures [9], muscle imaging [10,11], as well as electrophysiologic markers, including motor unit number estimate (MUNE) and compound motor action potential amplitude (CMAP) [12–14]. More recently, the technique of electrical impedance myography

(EIM) has also been studied in this disease. EIM is a bioimpedance-based procedure in which a high-frequency electrical current is applied to a localized area of muscle and the consequent voltages measured [15]. The voltages reflect changes in both muscle membrane health and size and well as local compositional changes to the muscle. A longitudinal study in children with mainly older SMA Type 2 and Type 3 patients has shown the relative stability of EIM values over time, in contrast to normal children who show increasing EIM phase values with age, consistent with progressive muscle growth and maturation [16]. Since EIM is non-invasive, painless, and simple to apply, it may be especially useful in young children and is currently being investigated as a part of a multicenter study.

In an effort to further refine and study EIM as a potential technology that can be readily applied for assessment of SMA therapies in both animals and humans, we undertook a study of the *Smn1^{c/c}* mouse with two main goals. First, we were interested in identifying differences in EIM, CMAP, and MUNE in a group of *Smn1^{c/c}* mice longitudinally over an extended period of time (up to 1 year of age). Second, we sought to determine how these measures correlated to serologic biomarkers, including SMN levels and histological data.

Methods

Animals

All procedures were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee (IACUC). Breeding colonies of *Smn1^{c/c}* mice (strain: B6.129-*Smn1^{tm5(Smn1/SMN2)Mrph/J}*) were established from animals obtained from Jackson Labs (Bangor, Maine). Animals were genotyped by tail snip.

Given that this animal model develops necrosis of the tail, pinna of ear, and digits of hind-paw up to an age of approximately P90, special care was mandated by our animal research facility.

Specifically, animals were not weaned until P28. Special bedding and gel-packs (DietGel 76A, PharmaSer, Framingham, MA) were provided at all times. As long as necrosis was present, the animal was provided meloxicam subcutaneously daily. Any animal developing paw (as compared to digital necrosis) or losing more than 20% body weight was euthanized. No electrophysiological or behavioral measurements were allowed until the necrosis had completely resolved, which was at approximately 15 weeks of age. A total of only 7 *Smn1^{c/c}* and 7 wild-type (WT) mice were ultimately studied for the full 15–52 week duration post necrosis and included in the longitudinal analysis. An additional 4 *Smn1^{c/c}* and corresponding 6 WT animals were ultimately also included and followed out to 52 weeks, at which time all animals were measured a final time and sacrificed.

Experimental design

There were two separate components to the study, the first being a longitudinal element, in which animals were followed at a regular intervals from 15 weeks of age until 1 year of age, and the second being a cross-sectional element, when the animals were all sacrificed at approximately 1 year of age. Body weight, front paw grip strength, CMAP, MUNE, and EIM were obtained on a regular basis for 37 weeks. At approximately 1 year of age all animals were sacrificed, at which time gastrocnemius muscle, spinal cord (L4–5), and whole blood were collected for further analysis.

Functional study

The front paw grip strength was measured by a grip strength meter single computerized sensor with standard pull bars (CAT # 1027CSM, Columbus Instruments). The animal was allowed to grasp a small bar connected to a sensitive force transducer. Holding the lower back of the animal, the investigator (JL) pulled the animal away from the bar until it lost its grip. The maximum force recorded out of 5 trials was recorded.

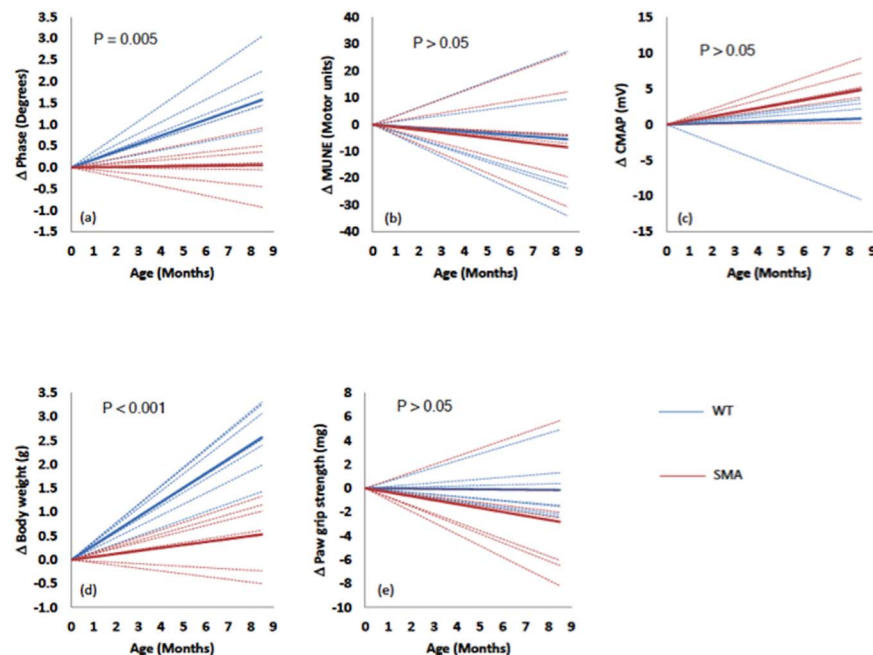


Figure 1. Trajectories of change (\pm standard deviation) over the period of 15 weeks to 52 weeks for the 7 *Smn1^{c/c}* animals (in black) and 7 WT animals (in gray) followed for the entire duration. Data is normalized to baseline with a least squares fit of the data. Only weight and EIM showed significant differences in the trajectories between the groups. doi:10.1371/journal.pone.0111428.g001

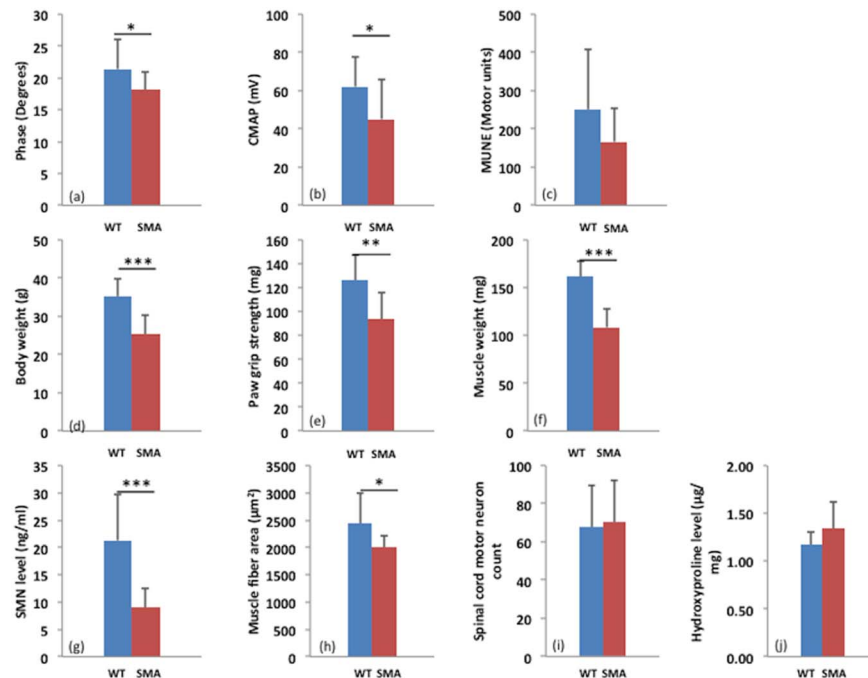


Figure 2. Column plots comparing 1-year end point data for WT and SMA animals. While most of the readily obtained measures, including body weight, paw grip strength, and muscle size are smaller and SMN is markedly reduced, all electrophysiological parameters show only modest differences. In addition, hydroxyproline, a measure of muscle fibrosis, is non-significantly increased in the SMA animals. * <0.05 , ** <0.01 , *** <0.001 . doi:10.1371/journal.pone.0111428.g002

CMAP and MUNE measurements

CMAP and MUNE were performed on the left hind limb via TECA Synergy T2 EMG Monitor System (Viasys, Madison, WI) while the animal was under anesthesia, as previously described [17]. Briefly, the sciatic nerve was supramaximally stimulated at the sciatic notch and the entire distal leg muscle compartment recorded via disposable ring electrodes with a ground electrode placed on the right hind paw to record the CMAP [18,19]. For MUNE, we followed a previously published incremental approach

for use in animals described by others [20,21]. Briefly, 10 incremental steps were recorded and averaged to determine the average single motor unit potential amplitude. The MUNE was calculated by dividing the CMAP amplitude by the average single motor unit potential amplitude. Of note, the MUNE analysis would have been more complete had multiple methods been used to confirm the MUNE, including multipoint [22] and modified multipoint [23]. However, these were not attempted given the already complex data set being collected; in addition, performing these methods in a mouse are extremely difficult given their small size and the need to move stimulating electrodes to different point along the nerve.

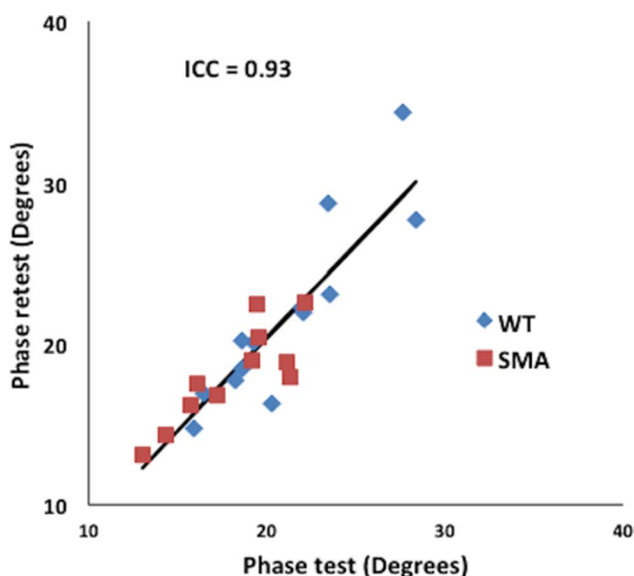


Figure 3. Test-retest reproducibility of EIM recorded from the mouse gastrocnemius at the one year end point. doi:10.1371/journal.pone.0111428.g003

EIM study

All EIM measurements were performed with the animals placed under 1% isoflurane anesthesia delivered through a nose cone with a heating pad underneath the limb to maintain consistent temperature. The leg was then taped to the measuring surface at an approximately 45° angle extending out from the body, away from the head.

A fixed 4-electrode array was placed over the gastrocnemius muscle, as previously described [17]. EIM measurements were performed with a Skulpt Inc EIM1103 system (San Francisco, CA).

Serological, pathological and histological studies

1. SMN protein concentration. The whole blood was obtained via cardio-puncture and preserved at -80°C . These samples were analyzed by PharmOptima with an electrochemiluminescence-based SMN immunoassay developed by PharmOptima (Portage, MI), using Meso Scale Discovery technology. 2. Muscle weight and myocyte fiber area. The entire gastrocnemius muscle was immediately excised at its proximal end just below the knee and cutting the gastrocnemius tendon distally, and its mass obtained.

The muscle was then snap-frozen in isopentane cooled in liquid nitrogen and stored at -80°C . The tissue was then cut into $5\ \mu\text{m}$ slices and stained with hematoxylin and eosin. Stereological measurements were made using a Zeiss Axiophot microscope with a motorized stage interfaced with a Dell Optiflex 380 computer running Stereo Investigator (MBF Biosciences, Inc., Williston, VT) software. Approximately 30 cells from each muscle were evaluated in a blinded fashion by a single investigator (TG). 3. **Hydroxyproline level.** In order to assess the quantity of connective tissue in the muscle as a consequence of disuse/neuronal loss, a commercially available assay (Kit #6017, Chondrex, Inc. Redmond, WA) was used to measure muscle hydroxyproline content. 4. **Spinal cord motor neuron number.** All animals were perfused and then fixed with 4% Formalin. To keep the spinal cord intact, the whole spinal column was removed and then decalcified by EDTA over several days. Then, the lumbar 4 and 5 levels of the spinal cord were removed, cut into $5\ \mu\text{m}$ section in paraffin, and stained by cresyl violet. Bilateral motor neuron number in the anterior horn on each section was counted blind to group designation. Regions of interest were counted at 40X magnification using a Zeiss Axiophot microscope interfaced with a Dell Optiflex 380 computer running Stereo Investigator (MF Biosciences, Inc, VT). The region of interest was sub-divided into sections using the optical fractionator within the Stereo Investigator software and examined individually. Inclusion of a cell as a motor neuron was based on cell size, centering of nucleus within the cell, and the amount of cytoplasm within the cell.

Data analysis

In order to utilize all the data obtained, differences between animals were initially assessed via repeated measures 2-way ANOVA, in which the independent variable was group and dependent variable the individual electrophysiological measures. However, in order to mirror standard practices in clinical trials, trajectories of change were then calculated for each individual animal over time (fit via linear regression anchored to the baseline visit), comparing the slopes of the trajectories for each of the measures between groups of animals. Unpaired t-tests and Pearson correlation analyses were also performed to determine the relationship between various functional, electrophysiological, pathological, and histological studies at the time of sacrifice at one year of age. For all statistical tests, significance was determined at $p < 0.05$, two-tailed. All results are summarized as mean \pm standard error.

Results

Differences between groups for the three main physiological measures: CMAP, MUNE, and EIM

In order to effectively capture all the data from multiple visits in the comparison between groups, we performed a two-way repeated measures ANOVA. There was a highly significant group effect for EIM ($F(12,130) = 44$, $p < 0.001$) and a similarly strongly significant group effect for CMAP ($F(12,130) = 26$, $p < 0.001$) but a non-significant group effect for MUNE ($F(12,130) = 0.31$, $p = 0.58$). A significant interaction term ($p < 0.001$) was also present for EIM but not MUNE or CMAP.

Longitudinal trajectories for all measures

As Figure 1 demonstrates, of the three electrophysiological parameters, only EIM demonstrated a significant difference over time in the SMA mice as compared to the WT mice, although the difference appeared to be due mainly to increasing values for the

WT animals rather than to a reduction in values in the SMA animals. Animal weight showed a similar trajectory.

End point-cross sectional study

Figure 2 provides a summary of the endpoint data for the two groups of animals. Figure 2a, 2b, and 2c show that body weight, front paw grip strength, and gastrocnemius muscle mass in the SMA animals were reduced compared to controls ($p < 0.01$ for all); of note, muscle mass was 32.8% lower in the SMA animals as compared to WT. Figure 2d, 2e and 2f show that there were borderline significant differences between the groups for both EIM ($p = 0.045$) and CMAP ($p = 0.043$); MUNE showed no significant difference between the groups.

Figure 2g, 2h, 2i and 2j showed the significant differences between the groups for both SMN concentration ($p < 0.001$) and myocyte area ($p = 0.043$); Spinal cord motor neuron count and hydroxyproline level did not show a significant difference between the groups.

Figure 3 shows test-retest repeatability of the endpoint data; as can be seen, the repeatability is good with an intra-class correlation coefficient of greater than 0.90.

End Point Correlations

The correlations are summarized in Table 1, with selected correlations provided in Figure 4. Among electrophysiological measures, only EIM showed the most consistent correlations with serological, pathological and histological measures, as presented in Figure 4a (SMN concentration, $r = 0.57$, $p = 0.003$), 4b (hydroxyproline level, $r = -0.50$, $p = 0.019$), and 4c (spinal cord motor neuron count, $r = 0.41$, $p = 0.047$), although it did not correlate significantly with muscle fiber size. Interestingly, EIM phase also correlated to CMAP (Figure 4d, $r = 0.44$, $p = 0.04$) whereas it had a poor correlation with MUNE (Figure 4e, $r = -0.33$, $p = 0.12$).

Discussion

Overall, the results of this study support that EIM is sensitive to disease status in $\text{Smn}1^{c/c}$ mice. Of the 3 electrophysiological biomarkers, both EIM and CMAP revealed significant differences between WT and SMA animals and only EIM revealed a difference in trajectory between the groups over the approximately 36-week period of study. This finding is analogous to the data obtained in our longitudinal study of older children with SMA [16]. While this EIM finding may appear useful, it is important to also point out that body weight showed a very similar trend (compare Figures 1a and 1c), which itself has been suggested as a useful measure in the $\text{SMN}\Delta 7$ mouse [24]. An obvious concern is that EIM is proving nothing more than a surrogate for body weight. In fact, it is not unreasonable to suspect that the two measures are associated. A major component of body weight is muscle mass, and EIM, while not a direct measure of muscle mass, is deeply impacted by the number and size of muscle fibers [25]. Since more, larger fibers imply greater muscle mass and hence greater body weight, it is not surprising that EIM and body weight show similar trends. Another point worth highlighting is that while the final measurements show a significantly lower CMAP for SMA animals than for WT (Figure 2), the WT CMAP actually decreased slightly over time whereas the SMA increased slightly (Figure 1). This apparent paradox is explained by the fact that the CMAP data in the SMA animals was lower throughout the study, and although both groups' values converged slightly over time, they never approached the point of equivalence.

Interestingly, there were no significant differences between the number of motor neurons in the spinal cords of the diseased mice

Table 1. Summary of correlations.

		Muscle weight	SMN	Hydroxy-proline	Muscle fiber area	Motor neuron count	CMAP	MUNE
SMN	r	0.62						
	p	0.00						
Hydroxy-proline	r	-0.41	-0.37					
	p	0.06	0.09					
Muscle fiber area	r	0.39	0.01	0.06				
	p	0.07	0.95	0.78				
Spinal cord motor neuron count	r	-0.05	0.03	-0.22	0.13			
	p	0.84	0.89	0.33	0.55			
CMAP	r	0.41	0.39	-0.36	0.36	0.34		
	p	0.08	0.07	0.12	0.12	0.13		
MUNE	r	0.11	0.29	0.09	0.16	0.07	0.13	
	p	0.62	0.17	0.68	0.47	0.76	0.55	
Phase	r	0.26	0.57	-0.50	0.07	0.41	0.44	-0.33
	p	0.25	0.00	0.02	0.75	0.05	0.04	0.12

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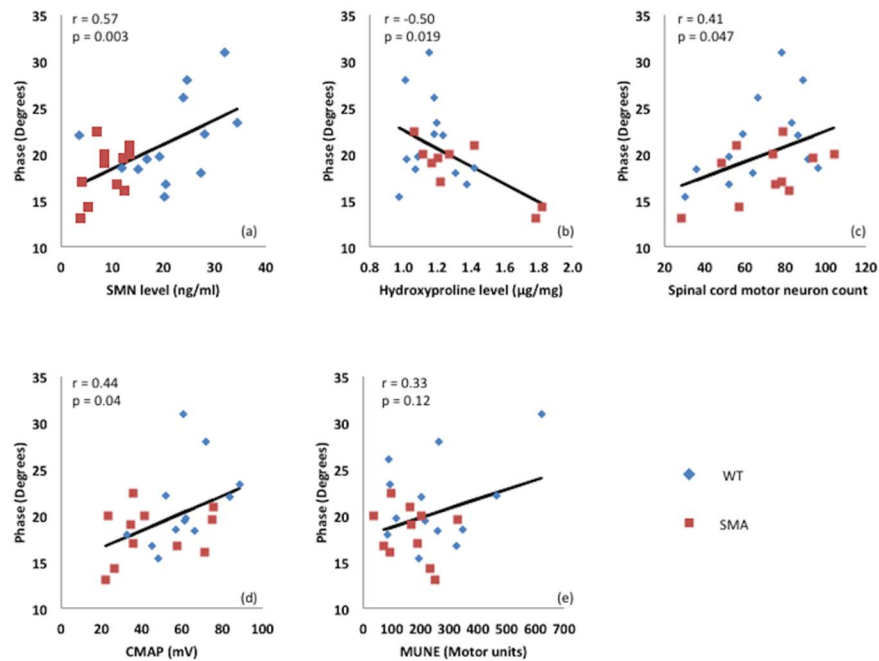


Figure 4. A selection of the correlations between various parameters for both animal types combined.
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versus the healthy mice, consistent with previous work showing a normal number of neurons in the L3 and L4 ventral roots [5]. This is in contrast to features of reduced MUNE and normalized CMAP because of collateral reinnervation that have been reported in patients with mild SMA [12]. This discordance between the animal model and the human disease is problematic and brings into question the value of this model. Still we do know that SMN is expressed in muscle as well [26], so it is possible that we are identifying muscle specific effects that may still be relevant to disease pathogenesis even if not primary motor neuron pathology. What remains surprising, however, is that despite this, EIM correlated to the motor neuron counts, perhaps suggesting some general relationship between EIM values and the number of motor neurons in a limb. It is also intriguing that EIM correlated to the SMN protein concentration, a second somewhat unexpected finding. Since SMN protein is expressed in muscle [26], it is possible that SMN levels are correlating closely with muscle size and weight, both of which are being captured in the EIM 50 kHz phase data.

How can these seemingly disparate results be integrated? Since there is no apparent loss of motor neurons, either by MUNE or motor neuron counting in the spinal cord, the mildly reduced pathology at the distal motor neuron or in the muscle itself. The substantially greater loss of muscle weight as compared to reduction in fiber size (34% vs. 18%) suggests that actual loss of individual muscle fibers (e.g., due to denervation) rather than just limited atrophy may be playing an important role in these findings. Loss of motor neuromuscular junctional input, with subsequent severe fiber atrophy, would be consistent with evidence that SMN plays an important role in the distal nerve and skeletal muscle [27,28]. Indeed, other work has shown that neuromuscular junctions are abnormal in this model [5]. Primary muscle abnormalities would have the effect of leaving MUNE unaltered while at the same time reducing the EIM and CMAP values [29], Further supporting this interpretation is the increase in hydroxy-

proline in the SMA animal muscle, suggesting increased connective tissue in the muscle, consistent with myopathic features that have been described in SMA [30].

A major limitation of this study is that of 220 SMA animals bred, only 11 ultimately could be studied due limitations placed on us by our institution's animal ethics committee. Inasmuch as the degree of necrosis parallels the degree of motor neuron loss, we may have evaluated only the most mildly affected animals. Second, the small number of animals studied, the large number of analyses performed, and the relatively modest p values for a number of the outcomes implies that the study is subject to both type 1 and type 2 error. Third, we only evaluated gastrocnemius, a mildly affected muscle in this model [31]. However, we have been developing approaches for measuring other muscles in the mouse including tibialis anterior, quadriceps, fore limb and even axial muscles using minute needle electrode arrays. Thus, this work can be viewed as only an initial effort toward assessing muscle condition in SMA mice using EIM. Fourth, we have only focused on a single frequency of electrical current in these studies; it is possible that multifrequency metrics could provide additional valuable data.

In summary, these data support that EIM may play a useful role in the evaluation of mild SMA animals and that EIM data correlate to some extent with other biomarkers. It also supports its potential application in the study of human SMA. However, it is clear that additional study of this and other models of SMA will be needed to fully understand the complex relationship between electrophysiological biomarkers and disease status.

Author Contributions

Conceived and designed the experiments: SR. Performed the experiments: JL TG. Analyzed the data: JL TG SR GR PZ. Contributed reagents/materials/analysis tools: JL PZ GR. Contributed to the writing of the manuscript: JL SR WA PZ. Other: WA GR PZ.

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