

Dysfunction of axonal membrane conductances in adolescents and young adults with spinal muscular atrophy

Michelle A. Farrar,^{1,2} Steve Vucic,^{1,3} Cindy S.-Y. Lin,^{1,4} Susanna B. Park,^{1,5} Heather M. Johnston,² Desirée du Sart,⁶ Hugh Bostock⁷ and Matthew C. Kiernan^{1,5}

1 Neuroscience Research Australia, Randwick, Sydney, Australia

2 Department of Neurology, Sydney Children's Hospital and School of Women's and Children's Health, University of New South Wales, Sydney, Australia

3 Department of Neurology, Westmead Hospital and Western Clinical School, University of Sydney, Sydney, Australia

4 School of Medical Sciences, University of New South Wales, Randwick, Sydney, Australia

5 Prince of Wales Clinical School, University of New South Wales, Randwick, Sydney, Australia

6 Molecular Genetics Laboratory, Victorian Clinical Genetics Services, Murdoch Children's Research Institute, Parkville, Victoria, Australia

7 Institute of Neurology, University College London, Queen Square, London, UK

Correspondence to: Professor Matthew C. Kiernan,
Neuroscience Research Australia,
Barker St, Randwick,
Sydney,
NSW 2031, Australia
E-mail: m.kiernan@unsw.edu.au

Spinal muscular atrophy is distinct among neurodegenerative conditions of the motor neuron, with onset in developing and maturing patients. Furthermore, the rate of degeneration appears to slow over time, at least in the milder forms. To investigate disease pathophysiology and potential adaptations, the present study utilized axonal excitability studies to provide insights into axonal biophysical properties and explored correlation with clinical severity. Multiple excitability indices (stimulus–response curve, strength–duration time constant, threshold electrotonus, current–threshold relationship and recovery cycle) were investigated in 25 genetically characterized adolescent and adult patients with spinal muscular atrophy, stimulating the median motor nerve at the wrist. Results were compared with 50 age-matched controls. The Medical Research Council sum score and Spinal Muscular Atrophy Functional Rating Scale were used to define the strength and motor functional status of patients with spinal muscular atrophy. In patients with spinal muscular atrophy, there were reductions in compound muscle action potential amplitude ($P < 0.0005$) associated with reduction in stimulus response slope ($P < 0.0005$), confirming significant axonal loss. In the patients with mild or ambulatory spinal muscular atrophy, there was reduction of peak amplitude without alteration in axonal excitability; in contrast, in the non-ambulatory or severe spinal muscular atrophy cohort prominent changes in axonal function were apparent. Specifically, there were steep changes in the early phase of hyperpolarization in threshold electrotonus ($P < 0.0005$) that correlated with clinical severity. Additionally, there were greater changes in depolarizing threshold electrotonus ($P < 0.0005$) and prolongation of the strength-duration time constant ($P = 0.001$). Mathematical modelling of the excitability changes obtained in patients with severe spinal muscular atrophy supported a mixed pathology comprising features of axonal degeneration and regeneration. The present study has provided novel insight into the pathophysiology of spinal muscular atrophy, with identification of functional abnormalities involving axonal K^+ and Na^+ conductances and alterations in passive membrane properties, the latter linked to the process of neurodegeneration.

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Keywords: spinal muscular atrophy; axon; excitability

Abbreviations: CMAP = compound motor action potential; SMAFRS = Spinal Muscular Atrophy Functional Rating Scale; SMN = survival motor neuron; τ SD = strength–duration time constant

Introduction

Spinal muscular atrophy is a disorder of spinal motor neurons characterized clinically by the development of muscle weakness and atrophy. With a carrier frequency of 1 in 50, spinal muscular atrophy is the leading genetic cause of infant death. Although mutations in the survival motor neuron 1 (*SMN1*) gene result in the spinal muscular atrophy phenotype, secondary to reduction in the SMN protein (Lefebvre *et al.*, 1995, 1997), the pathophysiological mechanisms remain to be elucidated. Specifically, while changes in SMN gene expression and splicing may induce disturbance of membrane transporters, axonal transport, and myelination pathways (Zhang *et al.*, 2008; Murray *et al.*, 2010), changes in axonal membrane function have not been investigated.

In terms of identifying the critical pathogenic processes and timeframes related to nerve degeneration in spinal muscular atrophy, there remain a number of unresolved issues (Farrar *et al.*, 2009). The clinical course may suggest a significant early loss of motor neurons, as manifested by a loss of strength or failure to gain strength with development. This may be followed by increasing stability of the surviving neurons with slow or absent clinical deterioration. However, an age dependent decline in compound muscle action potential (CMAP) amplitudes and motor unit number estimation values that correlated with functional decline has been previously demonstrated (Bromberg and Swoboda, 2002; Swoboda *et al.*, 2005; Lewelt *et al.*, 2010). Alternatively, CMAPs may remain stable in the face of a decline in motor unit number estimation values, suggesting lower motor neuron loss with compensatory collateral re-innervation (Swoboda *et al.*, 2005). In further contrast, a robust set of motor units may remain functional for decades (Bromberg *et al.*, 2003). The mechanisms underlying this spectrum of differential survival and potential compensation of motor neurons in spinal muscular atrophy remain unknown, but overall, these clinical changes seem very unusual for a neurodegenerative disorder.

In terms of previous pathophysiological approaches, studies of motor neurons in a spinal muscular atrophy mouse model have established hyperexcitability, with alterations in the distribution of ion channels at the cell membrane hypothesized (Mentis *et al.*, 2011). To gain insight into spinal muscular atrophy pathophysiology, protocols for testing of axonal membrane function have recently been developed for clinical assessment (Kiernan *et al.*, 2000). These techniques have been applied to neurodegenerative disorders including motor neuron disease, and have established functional abnormalities of persistent Na⁺ conductances and K⁺ ion channel function (Bostock *et al.*, 1995; Mogyoros *et al.*, 1998; Kanai *et al.*, 2006; Vucic and Kiernan, 2006). Upregulation of persistent Na⁺ conductances has been linked to the process of neurodegeneration and muscle cramping (Kanai *et al.*, 2003), both features of spinal muscular atrophy. As such, in an attempt to further clarify the pathophysiological mechanisms underlying

spinal muscular atrophy, the present study utilized axonal excitability techniques, measuring multiple excitability parameters in order to determine whether abnormalities in axonal membrane ion channel conductances were a feature of spinal muscular atrophy, and whether such abnormalities were linked to the process of neurodegeneration.

Materials and methods

Patients

Clinical and functional assessments were combined with conventional and specialized neurophysiological assessments in patients with spinal muscular atrophy types 2 and 3 who expressed homozygous *SMN1* deletions. By definition, patients with spinal muscular atrophy type 2 manifested weakness after 6 months of age with the ability to sit being the maximum motor milestone achieved. Patients with spinal muscular atrophy type 3 had symptomatic onset after 18 months of age and attained the ability to walk unaided (Munsat and Davies, 1992). All patients gave informed consent or assent to the procedures, which were approved by the South Eastern Sydney and Illawarra Area Health Service Human Research Ethics Committee. No patient had a history of diabetes or medication use known to potentially affect peripheral nerve excitability (Krishnan and Kiernan, 2005).

All patients were clinically assessed using the MRC Sum Score (Medical Research Council, 1976) and the Spinal Muscular Atrophy Functional Rating Scale (SMAFRS) (Elsheikh *et al.*, 2009). Specifically, the MRC sum score incorporated the MRC score of deltoid, biceps, wrist extensors, abductor pollicis brevis, iliopsoas, quadriceps femoris and tibialis anterior on both sides, with a normal strength score achieving a total of 70. The SMAFRS assessed the functional status and a score of 50 was deemed normal. Additionally, subjects were divided into two subgroups according to SMAFRS score: 'mild', SMAFRS > 40 or 'severe', SMAFRS score ≤ 40. This classification was chosen as a score >40 indicates ambulation or the ability to stand with assistance, whereas a score ≤40 indicates non-ambulatory patients.

In terms of genetic analysis, genomic DNA was isolated from blood in all patients with spinal muscular atrophy and homozygous *SMN1* deletions confirmed. Determination of *SMN2* copy number was undertaken using previously described techniques (McAndrew *et al.*, 1997; Smith *et al.*, 2007) to assess potential contribution as a disease-modifying component (Feldkotter *et al.*, 2002).

Nerve conduction and excitability studies

Conventional nerve conduction and specialized axonal excitability studies were undertaken in all patients with spinal muscular atrophy. Quantitative and qualitative single-unit electromyography was not routinely undertaken. EMG signals were amplified and filtered (3 Hz–3 kHz) using a GRASS ICP511 AC amplifier (Grass-Telefactor,

Astro-Med Inc.). Electronic noise was removed (Hum Bug 50/60 Hz Noise Eliminator, Quest Scientific Instruments) and sampled at 10 kHz using a 16-bit data acquisition card (National Instruments PCI-MIO-16E-4). Data acquisition and stimulation delivery was controlled by QTRACS software (©Institute of Neurology). Electrical stimulation was converted to current using an isolated linear bipolar constant current simulator (maximal output 50 mA; DS5, Digitimer). Temperature was measured with a surface probe at the wrist (Digitech, Jaycar).

Axonal excitability studies were performed using previously described threshold tracking protocols applied to the median nerve (Kiernan *et al.*, 2000). Briefly, the cathode was located over the median nerve at the wrist and the anode 10 cm proximally. The CMAP was recorded using surface electrodes (4620 M, Unomedical Ltd) positioned over the abductor pollicis brevis muscle with the active electrode at the motor point and the reference electrode 4 cm distal. Amplitude of the CMAP was measured from baseline to negative peak and the threshold tracking target set to 40% of the maximum, utilizing the area of steepest slope of the stimulus response curve. Threshold tracking follows the changes in the intensity of a test stimulus required to produce the target potential. Excitability testing incorporated a number of assessments designed to reflect different nodal and internodal properties, from which various excitability measures could be extracted for analysis. These included: (i) stimulus–response curves, which were determined using a 1 ms duration test stimulus increased from zero until supramaximal potentials were attained; (ii) strength–duration properties, which describe the stimulus strength required to excite nerves as stimulus width is increased from 0.2 to 1 ms duration (Weiss, 1901; Bostock, 1983; Mogyoros *et al.*, 1996); (iii) rheobase, defined as the threshold current for a stimulus of infinitely long duration was also calculated (Bostock *et al.*, 1998). Both parameters reflect activity of nodal persistent Na⁺ conductances (Mogyoros *et al.*, 1996; Bostock and Rothwell, 1997); (iv) threshold electrotonus, which measures internodal properties and conductances and provides an estimate of membrane potential. Prolonged 100 ms subthreshold currents were delivered and set to be +40% and +20% (depolarizing threshold electrotonus) and –40% and –20% (hyperpolarizing threshold electrotonus) of the control threshold current; (v) current–threshold relationship, which measures the rectifying properties of the axon (Bostock *et al.*, 1998; Kiernan *et al.*, 2000). The current–threshold relationship was assessed by measuring the change in threshold following the injection of polarizing currents of 200 ms duration, the strength of which was altered in 10% steps from +50% (depolarizing) to –100% (hyperpolarizing) of the control threshold; and (vi) the recovery cycle, which utilizes the effects of a supramaximal conditioning stimulus to measure changes in resting membrane potential at different conditioning–test intervals (decreasing from 200–2 ms) (Kiernan *et al.*, 1996, 2000). Three stimulus combinations were recorded: (i) unconditioned test stimulus (1 ms duration); (ii) supramaximal conditioning stimulus alone; and (iii) conditioning and test stimuli in combination. The response in (ii) was subtracted online from response in (iii) to eliminate contamination of the measured CMAP response by the supramaximal conditioning response at short interstimulus intervals. Ten stimulus combinations were repeated until four valid estimates were obtained. The following parameters were measured: (i) relative refractory period (ms), defined as the first intercept at which the recovery curve crosses the x-axis; (ii) superexcitability (%), defined as the largest reduction in threshold, peaking at a conditioning–test interval of <10 ms; and (iii) late subexcitability (%), defined as the largest increase in threshold following the superexcitability period after 10 ms.

Electrical model of nerve excitability

To model the excitability changes in motor axons in patients with spinal muscular atrophy and the effects of altered axonal conductances and passive membrane properties, mathematical simulations were undertaken using a model of the human axon (Bostock *et al.*, 1991, 1995; Kiernan *et al.*, 2005). Transient Na⁺ channels were modelled using the voltage-clamp data (Schwarz *et al.*, 1995), and persistent Na⁺ currents were added (Bostock and Rothwell, 1997). The equations for a single node and internode, representing a spatially uniform axon, were assessed by integration over successive small time steps (Euler's method); (Press *et al.*, 1992; Boland *et al.*, 2009). At times corresponding to those in human nerve excitability recordings, the excitability of the model nerve was tested repeatedly to determine threshold with an accuracy of 0.5%. The discrepancy between the thresholds determined for the model and those determined from a sample of real nerves was scored as the weighted sum of the error terms: $[(x_m - x_n)/s_n]^2$, where x_m is the threshold of the model, x_n the mean and s_n the standard deviation of the thresholds for the real nerves. The weights were the same for all threshold measurements of the same type (e.g. recovery cycle), and chosen to give an equal total weight to the different types of threshold measurement: current–threshold relationship, threshold electrotonus and the recovery cycle. The standard model was obtained by minimizing the discrepancy between the model and the normal control data with an iterative least squares procedure, so that alteration of any of the above parameters would make the discrepancy worse.

Data analysis

Peripheral nerve excitability parameters were compared with control data obtained from 50 age-matched subjects (28 males, 22 females; age range 8–39 years, mean 25.1, not significant). Controls were unrelated healthy volunteers recruited from the community. Further subgroup analysis of the normal control group was undertaken and demonstrated that there were no significant differences in axonal excitability parameters in mid-teen controls, compared to young adult controls. All controls gave informed consent or assent to the procedures, which were approved by the South Eastern Sydney and Illawarra Area Health Service Human Research Ethics Committee. While mean temperature was maintained >32°C in subjects with spinal muscular atrophy and controls, peripheral excitability measures were compensated for temperature before statistical analysis, using the relations established in controls (Kiernan *et al.*, 2001). All results were expressed as mean ± standard error of the mean (SEM). Data sets were tested for skew prior to the application of statistical tests. For each parameter of the multiple excitability measurements, the differences in means were tested with Student's unpaired *t*-test. Correlations between excitability and clinical assessments were analysed by Spearman's rank correlation coefficient. A *P* < 0.05 was considered to be statistically significant.

Results

Clinical features

The clinical and genetic features for all 25 patients with spinal muscular atrophy are summarized in Table 1. The study population included 9 males and 16 females (mean age 22.2 years, range 13–40 years; disease duration 5–40 years). There were 11 patients

Table 1 Clinical and genetic details of 25 patients with spinal muscular atrophy types 2 and 3

Patients with spinal muscular atrophy types 2 and 3	SMN2 copy number	Age (years)	SMAFRS	MRC Score	MRC abductor pollicis brevis	Age at loss of independent ambulation (years)	Age of presentation (years)
1	2	40	0	10	2		
2	2	27	0	23	2		
3	1	19	3	26	2		
4	3	28	6	26	3		
5	–	14	2	28	2		
6	3	21	8	29	3		
7	–	15	5	34	3		
8	3	20	8	35	3		
9	3	20	13	36	3		
10	3	13	14	38	4		
11	3	18	17	42	4		
Median		20.0	6.0	29.0	3.0		
12	2	39	0	25	3	9	2
13	3	17	16	38	4	6	1.5
14	3	19	20	40	4	11	1.5
15	–	15	14	44	4	11	2
16	3	18	24	46	4	7	1.5
17	3	15	29	47	5	14	3
18	2	16	41	50	5	14	4
19	–	31	45	51	5	Ambulant	12
20	3	16	47	56	5	Ambulant	2
21	–	24	47	58	5	Ambulant	11
22	3	36	47	60	5	Ambulant	3
23	3	19	49	62	5	Ambulant	14
24	3	26	48	63	5	Ambulant	12
25	3	22	49	66	5	Ambulant	8
Median		19.0	43.0	50.5	5.0		3.0

The patients were clinically graded using the SMAFRS, with a maximum score of 50 when there is no disability. Muscle strength was clinically assessed using the Medical Research Council (MRC) for the sum of the MRC score of deltoid, biceps, wrist extensors, abductor pollicis brevis, iliopsoas, quadriceps femoris and tibialis anterior on both sides and normal strength scores a total of 70. Dash indicates that formal testing of *SMN2* copy number was not undertaken at the time of the study.

with spinal muscular atrophy type 2 and 14 patients with spinal muscular atrophy type 3.

Overall, there was a broad spectrum of clinical severity, ranging from mild to profound weakness and functional impairment, as reflected by the variation in MRC score (range 10–66, median 40) and SMAFRS (range 0–49, median 16). Specifically, there was marked clinical variation among patients with spinal muscular atrophy type 3, with half requiring wheelchair assistance in childhood and adolescence and the remainder continuing to walk in adulthood with mild muscle weakness, such that spinal muscular atrophy type did not define current gross motor functional status. Additionally, there was an overlap of clinical severity among non-ambulatory subjects with spinal muscular atrophy type 2 and subjects with spinal muscular atrophy type 3. Overall, 32% of patients from the present cohort were ambulant or able to stand with assistance, with relatively high levels of function. In the non-ambulatory group, 89% of patients had undergone spinal fusion and 33% received nocturnal non-invasive ventilation. In patients with spinal muscular atrophy, symptomatic muscle cramps and fasciculations were either infrequent or absent at the time of investigation.

The age of onset and spinal muscular atrophy phenotype overlapped between patients with two or three copies of *SMN2*. Three of the four patients with two copies of *SMN2* were functionally the weakest, with strength significantly lower in patients with two copies than in patients with spinal muscular atrophy with three copies of *SMN2* (MRC score: two copies *SMN2* 26.3 ± 8.0 , three copies *SMN2*, 43.4 ± 3.8 , $P < 0.05$). The fourth and discordant patient with two copies of *SMN2* was Patient 18 (Table 1), a 16-year-old male who was independently ambulant until aged 14 years and presently maintained the ability to stand with support for transfers, highlighting potential individual variation in copy number and clinical phenotype and the potential for additional disease modifying factors.

Baseline conventional neurophysiological assessment

As may have been expected, CMAP amplitude was significantly reduced in the 25 subjects with spinal muscular atrophy when compared to controls (spinal muscular atrophy, 3.6 ± 1.1 mV; controls, 7.7 ± 1.1 mV, $P < 0.0005$). The threshold currents required

to elicit a response were similar in patients with spinal muscular atrophy and controls (spinal muscular atrophy, 3.4 ± 1.1 mV; controls, 3.6 ± 1.1 mV, not significant). The SMAFRS separated subjects into two distinct groups: eight were ambulant or able to stand with assistance (termed 'Mild') and 17 were non-ambulant (termed 'Severe'). The CMAP amplitude differed between the spinal muscular atrophy subgroups and controls (mild spinal muscular atrophy 5.5 ± 1.2 mV; severe spinal muscular atrophy 2.8 ± 1.1 mV, $F = 37.6$, $P < 0.0005$, Fig. 1).

Measures of axonal excitability

Similar to the variability in clinical and genetic features in the present cohort of patients with spinal muscular atrophy, a large range of changes in axonal excitability were observed. Analysis of the excitability measures revealed two distinct groups that

corresponded to functional status (Table 2). Accordingly, measures of axonal excitability were further examined according to disease severity, to provide further insight into pathophysiology.

Mild spinal muscular atrophy group

A striking finding was that, despite lower CMAP amplitudes in the mild spinal muscular atrophy subgroup, multiple measures of excitability appeared similar to controls (Table 2). Of relevance, all patients with spinal muscular atrophy in this group maintained MRC grade 5 strength of abductor pollicis brevis, the target muscle tested.

Severe spinal muscular atrophy group

The slope of the normalized stimulus–response curve was significantly reduced in spinal muscular atrophy (spinal muscular atrophy, 3.0 ± 1.1 ; controls, 4.7 ± 1.0 , $P < 0.0005$). In addition to a markedly reduced CMAP amplitude, this cohort of patients exhibited prominent excitability abnormalities (Table 2) as follows:

- (i) Strength duration properties. The strength-duration time constant (τ SD) serves as an indirect measure of nodal persistent Na^+ conductances (Bostock and Rothwell, 1997). The mean τ SD was significantly prolonged in patients with severe spinal muscular atrophy compared to controls (spinal muscular atrophy, 0.49 ± 0.02 ms; controls, 0.40 ± 0.01 ms, $P \leq 0.001$, Fig. 2A). As described in the 'Materials and methods' section, peripheral excitability measures were compensated for temperature prior to undertaking statistical analysis (spinal muscular atrophy $32.2 \pm 0.3^\circ\text{C}$; controls $32.9 \pm 0.2^\circ\text{C}$). The magnitude of difference for τ SD remained significant even without temperature correction (raw data: spinal muscular atrophy, 0.50 ± 0.02 ms; controls, 0.40 ± 0.01 ms, $P \leq 0.005$). Although there was no significant alteration in rheobase (spinal muscular atrophy,

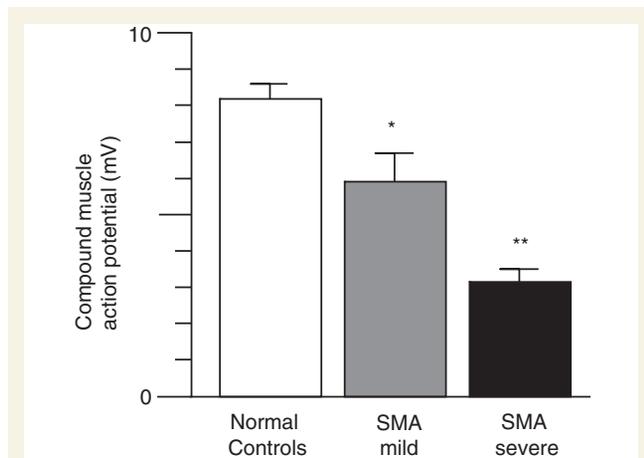


Figure 1 Mean data illustrating that the CMAP was significantly reduced in patients with spinal muscular atrophy compared to controls (* $P < 0.05$, ** $P < 0.0001$).

Table 2 Indices in multiple excitability measurements

	Normal (n = 50)	Spinal muscular atrophy		
		All (n = 25)	Mild (n = 8)	Severe (n = 17)
τ SD (ms)	0.40 ± 0.01	$0.47 \pm 0.02^*$	0.39 ± 0.02	$0.49 \pm 0.02^{***}$
Rheobase (mA)	2.5 ± 1.1	2.3 ± 1.1	2.7 ± 1.1	2.2 ± 1.1
TEd ₄₀ (peak) (%)	67.4 ± 0.7	$70.6 \pm 0.1^*$	68.3 ± 1.5	$71.6 \pm 1.1^{**}$
TEd ₄₀ (90–100 ms) (%)	45.0 ± 0.6	46.6 ± 1.3	44.1 ± 0.5	$48.3 \pm 1.9^*$
TEd ₂₀ (peak) (%)	36.8 ± 1.0	$44.4 \pm 2.3^{**}$	37.6 ± 1.6	$48.6 \pm 2.6^{****}$
TEh ₄₀ (20–40 ms) (%)	-91.2 ± 1.3	$-102.7 \pm 3.6^{***}$	-86.7 ± 2.4	$-111.9 \pm 3.9^{****}$
TEh ₄₀ (90–100 ms) (%)	-117.2 ± 2.4	-121.7 ± 5.3	-106.9 ± 5.8	$-131.4 \pm 6.8^*$
TEd ₄₀ (undershoot) (%)	-19.3 ± 0.6	$-23.6 \pm 1.4^{**}$	-18.9 ± 1.2	$-24.4 \pm 1.7^{****}$
TEh ₄₀ (overshoot) (%)	16.1 ± 0.6	$24.2 \pm 1.6^{****}$	17.7 ± 1.5	$26.9 \pm 1.7^{****}$
Superexcitability (%)	-25.9 ± 0.8	-26.6 ± 1.6	-27.7 ± 1.8	-26.6 ± 2.3
Subexcitability (%)	14.9 ± 0.6	$18.4 \pm 0.9^{**}$	15.7 ± 0.8	$20.2 \pm 1.2^{****}$
Resting current-threshold gradient	0.61 ± 0.01	$0.56 \pm 0.03^*$	0.6 ± 0.03	$0.51 \pm 0.03^{**}$

Values are represented as mean \pm SEM.

τ SD = strength duration time constant; TEd = depolarizing threshold electrotonus; TEh = hyperpolarizing threshold electrotonus. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$, compared with normal values.

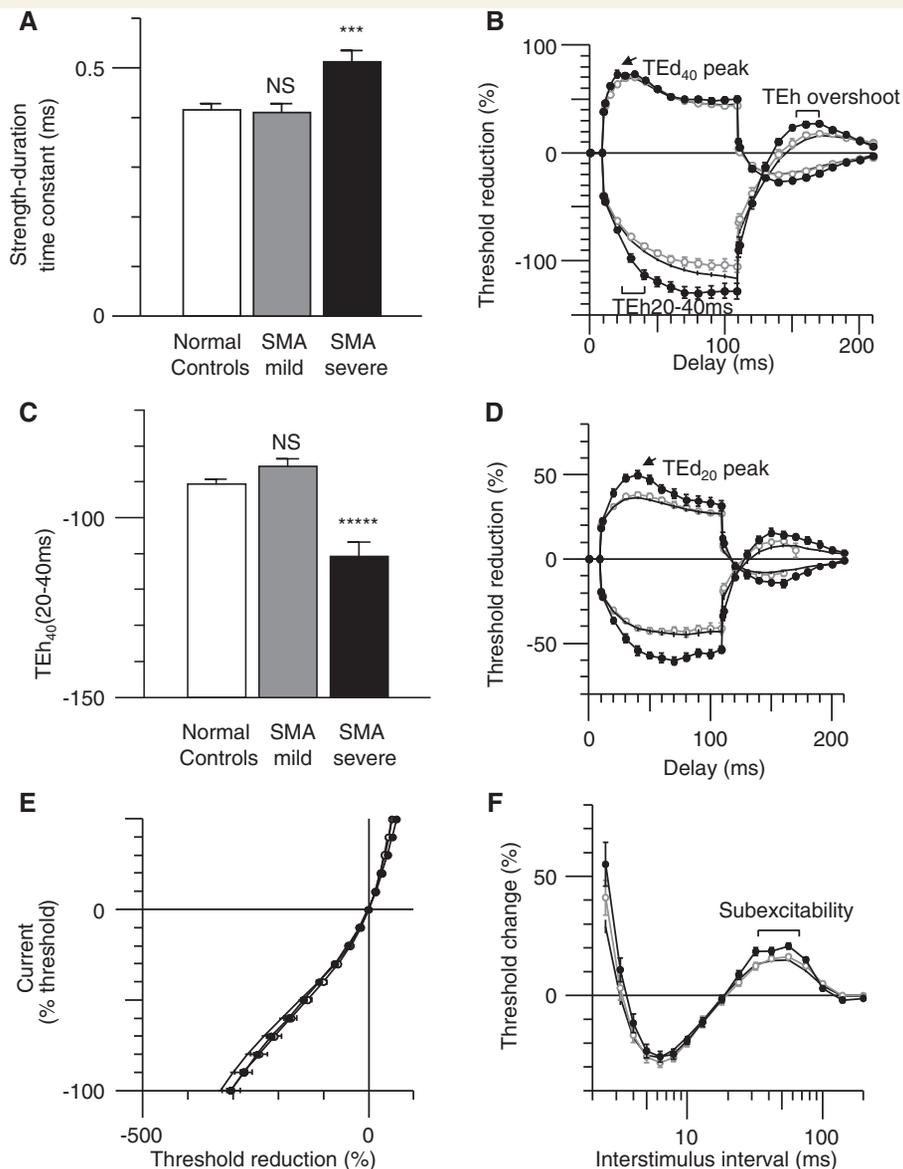


Figure 2 Comparison of multiple measures axonal excitability in the mild and severe spinal muscular atrophy subgroups and normal controls. Mean and standard errors of controls (white, black line), mild spinal muscular atrophy subgroup (grey, unfilled circles) and severe spinal muscular atrophy subgroup (black, filled circles) are plotted. (A) Strength–duration time constant (τ_{SD}) ($***P < 0.001$). (B) Changes in threshold during and after 40% polarization of unconditioned threshold. Threshold reduction with depolarization is represented in an upward direction and hyperpolarization in a downward direction. (C) Group data revealed a significant increase in threshold to sub-threshold 40% hyperpolarizing currents at 20–40 ms ($*****P < 0.0001$). (D) Changes in threshold during and after 20% polarization of unconditioned threshold. (E) The current threshold relationship; patients with spinal muscular atrophy had a tendency to greater threshold changes to depolarizing and weak hyperpolarizing currents, consistent with the early ‘fanning out’ in threshold electrotonus and smaller changes in response to stronger hyperpolarizing currents. (F) Recovery cycle of excitability. The most significantly altered excitability parameters in the severe spinal muscular atrophy group included hyperpolarizing threshold electrotonus_{40(20–40)} ($P < 0.0005$), depolarizing threshold electrotonus_{40(peak)} ($P < 0.01$), depolarizing threshold electrotonus_{20(peak)} ($P < 0.0005$), hyperpolarizing threshold electrotonus_{40(overshoot)} ($P < 0.0005$) and late subexcitability ($P < 0.0005$) and are indicated on the figures. TE_d = depolarizing threshold electrotonus; TE_h = hyperpolarizing threshold electrotonus.

2.2 ± 1.1 mA; controls, 2.5 ± 1.1 mA, not significant), the previously established negative correlation between τ_{SD} and rheobase in healthy controls was maintained in this cohort of patients with spinal muscular atrophy ($R = -0.3$, $P < 0.05$) (Mogyoros et al., 1998).

(ii) Threshold electrotonus. Long subthreshold currents applied during threshold electrotonus spread from the node into the internode and accordingly excitability changes reflect inter-nodal properties and conductances in addition to providing an estimate of resting membrane potential. Threshold

electrotonus recordings in patients with severe spinal muscular atrophy demonstrated steep changes during the early phases of hyperpolarization and depolarization [hyperpolarizing threshold electrotonus₄₀(20–40) spinal muscular atrophy, $-111.9 \pm 3.9\%$; controls, $-91.2 \pm 1.3\%$, $P < 0.0005$; depolarizing threshold electrotonus₄₀(peak) spinal muscular atrophy, $71.6 \pm 1.1\%$; controls, $67.4 \pm 0.7\%$, $P < 0.01$; depolarizing threshold electrotonus₂₀(peak): spinal muscular atrophy, $48.6 \pm 2.6\%$; controls, $36.8 \pm 1.0\%$, $P < 0.0005$, Fig. 2B–D]. These changes have been described as ‘fanning-out’, related to their resemblance to a Japanese fan (Kaji, 1997). The early fanning out was partially normalized during hyperpolarization, suggesting more accommodation due to activation of inward rectification by the hyperpolarization activated current I_h [hyperpolarizing threshold electrotonus₄₀(90–100): spinal muscular atrophy, $-131.4 \pm 6.8\%$; controls, $-117.2 \pm 2.4\%$, $P \leq 0.01$]. The accommodative response to 40% depolarizing currents during threshold electrotonus was complicated by some axons being excited and activating nodal slow K^+ channels (Trevillion *et al.*, 2007), and overall there was little change [depolarizing threshold electrotonus₄₀(40–60): spinal muscular atrophy $51.7 \pm 2.0\%$, controls, $51.3 \pm 0.7\%$, not significant; depolarizing threshold electrotonus₄₀(Accom): spinal muscular atrophy, $26.6 \pm 1.4\%$, controls, $22.1 \pm 0.6\%$, $P \leq 0.001$; depolarizing threshold electrotonus₄₀(90–100): spinal muscular atrophy, $48.3 \pm 1.9\%$, controls, $45.0 \pm 0.6\%$, $P < 0.05$]. The threshold electrotonus overshoot and undershoot were significantly increased in spinal muscular atrophy [depolarizing threshold electrotonus₄₀(undershoot): spinal muscular atrophy, $-24.4 \pm 1.7\%$; controls, $-19.3 \pm 0.6\%$, $P < 0.0005$; hyperpolarizing threshold electrotonus₄₀(overshoot) spinal muscular atrophy, $26.9 \pm 1.7\%$; controls, $16.1 \pm 0.6\%$, $P < 0.0005$].

- (iii) Current–threshold relationship. The current–threshold relationship estimates rectifying properties of nodal and internodal segments of the axon (Bostock *et al.*, 1998). Additionally, the slope of the curve was used to provide an estimate of the threshold analogue of input conductance (Kiernan *et al.*, 2000). The resting current–threshold relationship slope was significantly reduced in subjects with spinal muscular atrophy [resting current–threshold relationship gradient; spinal muscular atrophy, 0.51 ± 0.03 ; controls, 0.61 ± 0.01 , $P \leq 0.005$], suggesting reduced membrane conductance at the resting membrane potential (Fig. 2E).
- (iv) Recovery cycle of excitability. The recovery cycle reflects changes in membrane excitability in response to a supra-maximal conditioning stimulus (Kiernan *et al.*, 1996). The initial phase of the recovery cycle, the refractory period, reflects inactivation of transient Na^+ channels. The duration of the relative refractory period was not significantly different when compared to controls (spinal muscular atrophy, 3.1 ± 1.1 ms; controls, 2.9 ± 1.0 ms, not significant). Superexcitability, a period of increased axonal excitability due to a depolarizing after potential spreading to the internodal axolemma (Barrett and Barrett, 1982), was not significantly different in patients with spinal muscular atrophy

compared to controls (spinal muscular atrophy, $-26.6 \pm 2.3\%$; controls, $-25.9 \pm 0.8\%$, not significant). The final phase of the recovery cycle, referred to as the late subexcitability period, probably reflects activation of slow K^+ channels (Waxman and Ritchie, 1993; Kiernan *et al.*, 1996), and was significantly increased when compared to controls (spinal muscular atrophy, $20.2 \pm 1.2\%$; controls, $14.9 \pm 0.6\%$, $P < 0.0005$, Fig. 2F).

Reproducibility of excitability measurements

To determine intra-subject variability of excitability measurements in patients with spinal muscular atrophy, successive recordings were undertaken in patients without moving the electrodes. The strength–duration properties, threshold electrotonus, current–threshold relationship and recovery cycle of excitability graphs were almost identical. Excitability parameters were highly reproducible without significant variation, similar to previous studies in control subjects (Kiernan *et al.*, 2000; Tomlinson *et al.*, 2010a).

Correlations with strength and motor function

Combining measures of axonal excitability and clinical parameters, it was evident that specific excitability parameters were significantly associated with strength and overall function. Furthermore, given that patients with spinal muscular atrophy with lower *SMN2* copy number had less strength, it followed that *SMN2* copy number was related to strength and function (MRC, $R = 0.58$, $P = 0.01$; SMAFRS, $R = 0.54$, $P \leq 0.05$), supporting the relevance of excitability measures. A greater hyperpolarizing threshold electrotonus (20–40) was associated with decreased strength and function (MRC, $R = 0.69$, $P < 0.0005$; SMAFRS, $R = 0.76$, $P < 0.0005$, Fig. 3A; *SMN2* copy number, $R = 0.62$, $P < 0.05$). Additionally, a prolonged τ_{SD} was correlated with a reduction in the MRC sum score ($R = -0.50$, $P \leq 0.01$) and SMAFRS ($R = -0.51$, $P \leq 0.005$, Fig. 3B). Interestingly the τ_{SD} also correlated with the CMAP amplitude ($R = -0.45$, $P < 0.05$). Importantly, clinical measures of severity were also significantly correlated with neurophysiological measures of peripheral disease burden, namely CMAP amplitude (MRC, $R = 0.73$, $P \leq 0.0001$; SMAFRS, $R = 0.62$, $P < 0.005$, Fig. 3C; *SMN2* copy number $R = 0.51$, $P < 0.05$) and stimulus–response slope (MRC, $R = 0.67$, $P \leq 0.001$; SMAFRS, $R = 0.68$, $P < 0.0005$). The effects of age and axonal excitability were also assessed, and one variable strongly correlated with age: depolarizing threshold electrotonus (40–60 ms) $R = 0.76$, $P < 0.0005$ (Fig. 4).

Mathematical modelling of abnormal excitability properties

To assist in interpreting the complex changes observed in the clinical nerve excitability measures, a mathematical model of the

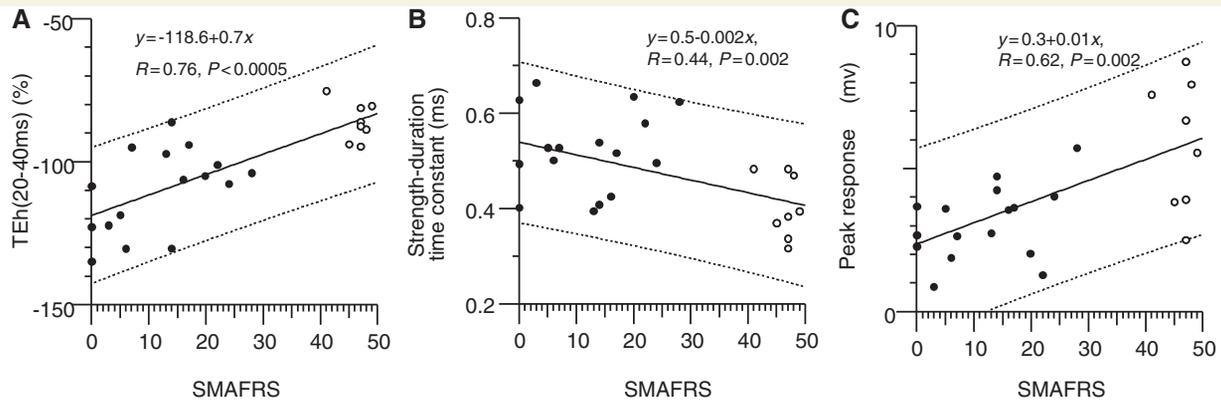


Figure 3 The relationships between axonal excitability parameters and clinical measures of severity: (A) SMAFRS and hyperpolarizing threshold electrotonus₄₀(20–40) ms; (B) SMAFRS and strength-duration time constant; (C) SMAFRS and CMAP. Means and 95% confidence limits for linear regression of mild spinal muscular atrophy subgroup (unfilled circles) and severe spinal muscular atrophy subgroup (filled circles) are plotted. TE_d = depolarizing threshold electrotonus; TE_h = hyperpolarizing threshold electrotonus.

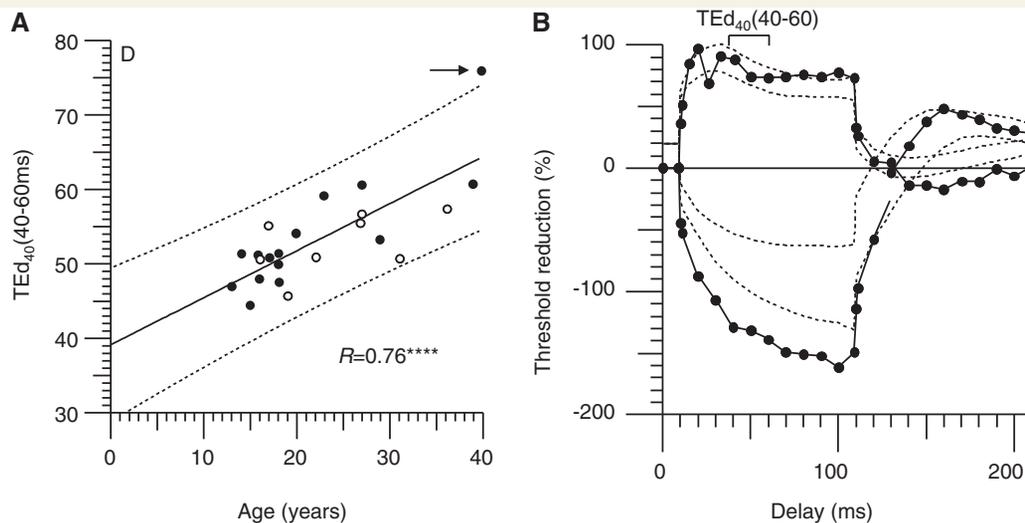


Figure 4 The effects of age and axonal excitability. (A) The change in threshold to sub-threshold 40% depolarizing currents at 40–60 ms, depolarizing threshold electrotonus (40–60 ms), a measure of slow K⁺ conductances, was the only measure that correlated with age. Patients in the mild spinal muscular atrophy subgroup are represented by unfilled circles and those in the severe spinal muscular atrophy subgroup by filled circles. The patient indicated by the arrow is the eldest and the one with the lowest MRC score. (B) Threshold electrotonus for the eldest patient (filled black circles) and 95% confidence intervals for normal controls (dashed lines). TE_d = depolarizing threshold electrotonus.

human motor axon was adjusted to provide a close match to the spinal muscular atrophy patient recordings. Since all patients with severe spinal muscular atrophy showed a similar pattern of abnormality, the most severely affected patients were selected for mathematical modelling across four levels of threshold electrotonus. Depolarizing threshold electrotonus₄₀ was then excluded as the polarizing current excited some axons, complicating the recordings.

Taken in isolation, no single parameter change could account for the spinal muscular atrophy recordings satisfactorily. The best match was a large reduction in internodal fast K⁺ channels (from 110–19.8 units), which reduced the discrepancy by 44% and

depolarized the axons by 1.3 mV. A better fit was obtained by changing two parameters and included reducing the internodal length by 13%, and reducing internodal fast K⁺ channels from 110 to 34 units. These two changes reduced the discrepancy by 55.8% and because there was less reduction in fast K⁺ channels, the axons were only depolarized by 1.0 mV.

However, the best fit and most plausible model to explain the recordings from patients with spinal muscular atrophy was determined by altering three parameters as follows: internodal length was reduced by 25%, internodal fast K⁺ channels were reduced from 110 to 5.75 units, while nodal fast K⁺ channels were increased from 19 to 34 units. These three changes reduced the

overall discrepancy by 71.8% and involved a membrane depolarization of 1.2 mV. Simulating the full axonal excitability recordings with this model is depicted in Fig. 5. Overall interpretation incorporating mathematical modelling suggested a combination of changes in passive cable properties and axonal ion channel function, with a reduction in fast K^+ channels as the most important channel alteration.

Discussion

The present study has established dysfunction of axonal conductances and passive membrane properties in patients with spinal muscular atrophy. In a mild cohort of patients with spinal muscular atrophy, there was reduction of peak amplitude without specific alterations in axonal excitability. In contrast, excitability measures were most abnormal in clinically severely affected patients with spinal muscular atrophy. This spectrum of change may suggest that the excitability changes reflected the progression of neurodegeneration and axonal loss. The interpretation of these latter changes will form the basis of discussion, in relation to pathogenic processes and potential timeframes related to neurodegeneration in spinal muscular atrophy.

Prior to analysing findings from the present study, a potential limitation of axonal excitability studies relates to the fact that only surviving axons may be assessed. As such, longitudinal data may provide clear understanding into the pathophysiology of surviving motor neurons and their capacity for re-innervation. However, results from the present study established that nerve excitability studies were feasible, valid, reproducible and reliable in spinal muscular atrophy, with all patients successfully completing this non-invasive procedure. Furthermore, the strong correlation of excitability with clinical and functional measures suggests the potential for nerve excitability to be developed as a marker of spinal muscular atrophy severity, for incorporation into assessment of future treatment approaches.

The *SMN2* gene is an almost identical copy of *SMN1* and produces a low level of full length and functional *SMN* transcripts. *SMN2* gene copy number varies between individuals and modulates the clinical phenotype, being inversely related to disease severity (Wirth *et al.*, 1999; Feldkotter *et al.*, 2002; Mailman *et al.*, 2002). The determination of *SMN2* copy number in the present series did not precisely predict spinal muscular atrophy type or age of onset, yet correlated with the clinical phenotype, suggesting that a higher copy number may be relevant to long-term progress. Interpretation may, however, be limited as the majority of patients

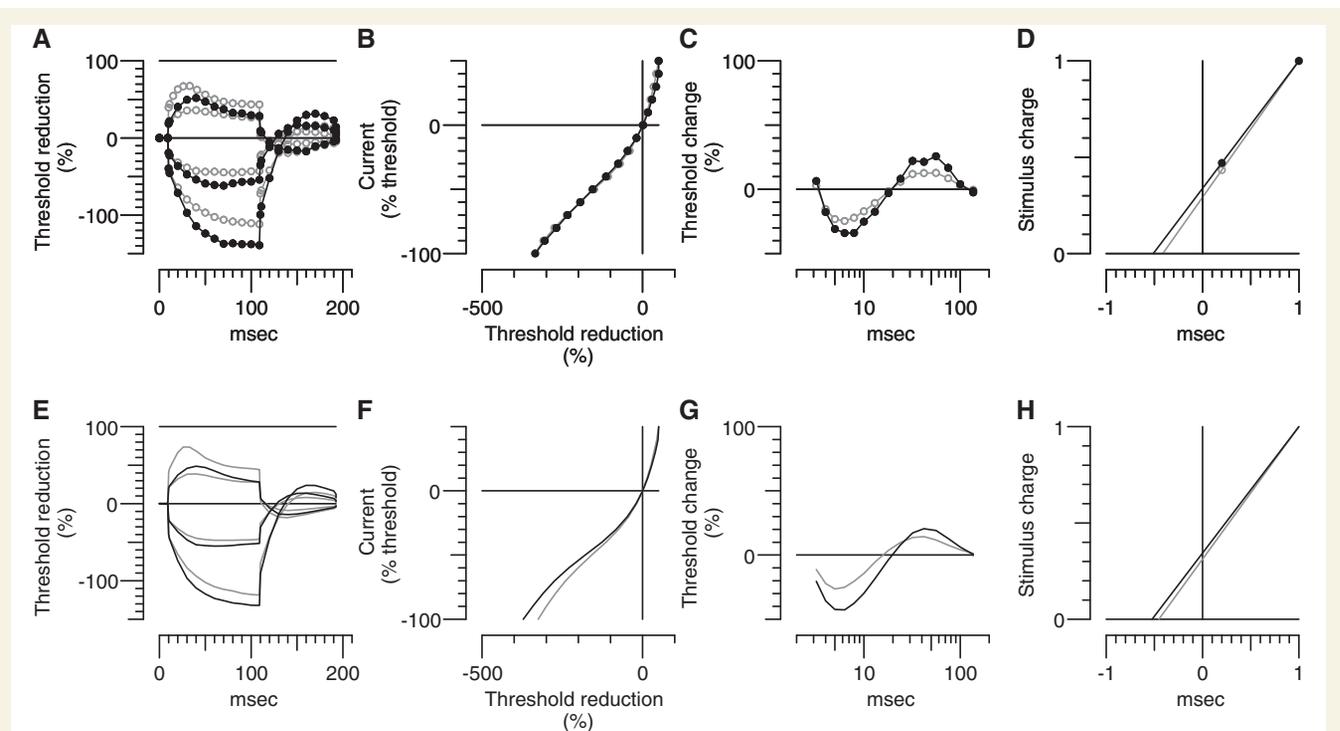


Figure 5 Simulation of the excitability changes in patients with spinal muscular atrophy with the mathematical model. (*Top*) Measures of axonal excitability for the most severely affected patients with spinal muscular atrophy (black filled circles) and normal controls (grey unfilled circles). (*Bottom*) Model of the excitability changes in patients with spinal muscular atrophy. Grey lines represent the standard model of human motor axons and black lines were generated by the model with internodal length reduced by 25%, internodal fast K^+ channels reduced from 110–5.75 units and nodal fast K^+ channels increased from 19–34 units. These alterations fitted to the most severely affected patients with spinal muscular atrophy, reduced the discrepancy by 71.8% and involved a membrane depolarization of 1.2 mV. (A, E) Threshold electrotonus for 100 ms polarizing currents $\pm 20\%$ and -40% of the resting threshold. (B, F) Current–threshold relationship, (C, G) recovery cycles and (D, H) charge–duration plot based on stimuli of 0.2 and 1 ms duration with the negative intercept on the x-axis equating to the strength–duration time constant, and the slope equal to the rheobase.

with spinal muscular atrophy had three copies of *SMN2* and predicting outcomes in individual cases is complex. Previous studies have suggested that not all *SMN2* genes are equivalent and that additional disease-modifying factors appear important in the pathogenesis of spinal muscular atrophy (Prior *et al.*, 2009). As such, these findings highlight the requirement to develop further biomarkers of disease severity, to improve current understanding of the pathophysiological mechanisms.

Alterations in potassium conductances and membrane potential in spinal muscular atrophy

Significantly increased threshold changes in depolarizing threshold electrotonus were established in patients with severe spinal muscular atrophy from the present study, most likely reflecting a reduction in fast K⁺ conductances. Similar effects have been described in relation to reduction of fast K⁺ conductances in animal studies utilizing 4-aminopyridine (Baker *et al.*, 1987), a blocker of fast K⁺ channels. Support also comes from studies of patients with episodic ataxia type 1, related to mutations in the *KCNA1* gene encoding the α -subunit of the fast K⁺ channel Kv1.1 (Tomlinson *et al.*, 2010b). Reduction in fast K⁺ conductances results in membrane depolarization and shifts slow K⁺ channels to a steeper segment of their activation curve, perhaps underlying the increases established in late subexcitability (Kiernan and Bostock, 2000). Paradoxically, superexcitability in subjects with spinal muscular atrophy was not altered, possibly reflecting complex changes that developed in passive membrane properties, as suggested by the results of mathematical modelling.

Separately, a progressive reduction in slow K⁺ conductances has been identified in patients with spinal muscular atrophy from the present study. Importantly, loss of K⁺ conductances and subsequent membrane depolarization has been observed during motoneuronal degeneration in amyotrophic lateral sclerosis (Kanai *et al.*, 2006; Vucic and Kiernan, 2006) and Wallerian degeneration (Moldovan *et al.*, 2009). These novel findings in patients with spinal muscular atrophy are also consistent with a mouse model of spinal muscular atrophy, demonstrating pre-messenger RNA splicing defects in the transcripts of genes related to voltage-gated potassium channels and also axonal transport processes (Zhang *et al.*, 2008). Further contributions may be derived from disruption of actin cytoskeletal dynamics and axonal transport processes that may also contribute to the pathophysiology of spinal muscular atrophy (Zhang *et al.*, 2003; Bowerman *et al.*, 2010).

Persistent sodium conductances in spinal muscular atrophy

The present study has established prolongation of τ SD in patients with spinal muscular atrophy, which correlated with reduced muscle strength and function. τ SD is an indirect measure of persistent Na⁺ conductances, which are voltage-dependent and active at resting membrane potential. Since the resting membrane potential appears depolarized in spinal muscular atrophy (related to loss of fast K⁺ channels as indicated by the modelling), such

changes may be at least part of the reason for the increase in τ SD. Separately, an increase in τ SD may occur during axonal regeneration or reinnervation (Ritchie, 1982; Kanai *et al.*, 2003; Nakata *et al.*, 2008; Sawai *et al.*, 2008), related to an increase in the number or overall conductance of persistent Na⁺ channels (Ritchie, 1982; Nakata *et al.*, 2008). The capacity for axonal sprouting in spinal muscular atrophy may be supported by clinical observation, as prolonged periods of stability may occur with partial compensation for concurrent motoneuronal degeneration. In further support, electromyography has demonstrated increasing features of re-innervation during the course of the disease (Hausmanowa-Petrusewicz and Karwanacuteska, 1986). Of further relevance, quantitative single unit electromyography data has demonstrated the presence of small, normal and enlarged motor units in patients with spinal muscular atrophy, further supporting mixed pathology comprising axonal degeneration and regeneration (Galea *et al.*, 2001). Alternatively, a prolonged τ SD may occur secondary to paranodal demyelination or changes in nerve geometry precipitated by axonal loss. As there was no evidence of demyelination in motor nerves of spinal muscular atrophy, this would seem an unlikely explanation. Furthermore, assessment of rheobase, a marker sensitive to changes in nerve geometry, was not significantly affected and the negative correlation between the τ SD and rheobase, as observed in normal controls, was also preserved in spinal muscular atrophy.

The prolonged τ SD in spinal muscular atrophy would be in keeping with previous studies of diseased motor neurons, including amyotrophic lateral sclerosis, Kennedy Disease and Machado Joseph Disease (Kanai *et al.*, 2003, 2006; Vucic and Kiernan, 2006, 2007). An increase in nodal persistent Na⁺ conductances in these conditions may underlie membrane instability and lead to spontaneous activity such as fasciculations and cramp (Kiernan *et al.*, 2011). In contrast to amyotrophic lateral sclerosis (Kanai *et al.*, 2006), the increase in τ SD was not identified in patients with spinal muscular atrophy with mild disease but was associated with clinical severity, suggesting that it would be unlikely to trigger neurodegeneration in spinal muscular atrophy. A further contrast to amyotrophic lateral sclerosis is that fasciculations and cramp are not frequently encountered in early spinal muscular atrophy (Kanai *et al.*, 2003), although these symptoms may become more prominent during the course of disease (Hausmanowa-Petrusewicz and Karwanacuteska, 1986). Importantly, upregulation of persistent Na⁺ conductances may provide insight into the mechanisms of neurodegeneration in spinal muscular atrophy. Specifically, the accumulation of intra-axonal Na⁺ may trigger reverse operation of the Na⁺–Ca²⁺ exchanger and activation of calcium-dependent enzyme systems such as calpain, phospholipases and protein kinase C.

Alterations in passive membrane properties in spinal muscular atrophy

Pathological studies in spinal muscular atrophy have demonstrated a reduction in axonal diameter, thinly myelinated fibres and distal axonal sprouting (Chien and Nonaka, 1989). Further, axonal regeneration is associated with shortened internodes (Ritchie, 1982).

The steep changes observed during hyperpolarizing threshold electrotonus were consistent with ‘fanning-out’ (Kaji, 1997) and may support alterations in passive membrane properties related to smaller axonal diameter, shortened internodes and thinned myelin. These structural alterations would reduce the capacitance of the internodal membrane and the initial rate of change of the internodal potential ($dv/dt = C/i$) would become greater, to induce fanning out of threshold electrotonus waveforms. Previous axonal excitability studies in regenerated nerve fibres and immature nerves have demonstrated an early fanning out of hyperpolarizing threshold electrotonus, reflecting a smaller axonal diameter and relative reduction in internodal capacitance (Yang *et al.*, 2000; Nodera *et al.*, 2004; Sawai *et al.*, 2008).

The degenerative process in spinal muscular atrophy may be predicted to affect larger diameter axons initially, progressing to intermediate and smaller diameter axons. Importantly, the excitability changes observed in the patients with spinal muscular atrophy from the present study were not solely attributable to those expected in smaller diameter axons (Shibuta *et al.*, 2010), which may be associated with reduced internodal lengths (Ibrahim *et al.*, 1995) and distinctive distributions of K^+ conductances (Fox and Ruan, 1989). As such, these findings further strengthen the interpretation that alterations in membrane conductances identified in the present study were not simply markers of disease progression, but reflected separate pathological or adaptive processes, either contributing to the degenerative process or preserving function in the face of diminishing motor unit number. As such, these processes may therefore serve as potential targets for pharmacotherapy.

The pathophysiology of spinal muscular atrophy, clinical context and significance

Axonal excitability measures have established dysfunction of axonal conductances and passive membrane properties in patients with spinal muscular atrophy. Short internodes are indicative of axonal regeneration, while the loss of K^+ channels appears to be a feature of neurodegeneration, supporting a mixed pathology of degeneration and regeneration. Importantly, excitability changes were abnormal in the most clinically affected patients, critically indicating that the excitability changes relate to the process of neurodegeneration and compensatory partial regeneration.

The clinical course of spinal muscular atrophy suggests a substantial early loss of motor neurons followed by increasing stability of the surviving neurons with slow or no clinical deterioration. This suggestion may be supported by the striking finding of normal axonal excitability in the patients with mild spinal muscular atrophy from the present study, despite reduction in CMAP. Such amplitude reductions may reflect loss of vulnerable motor neurons, with relatively preserved function in the surviving axonal population. Similar findings have been demonstrated in amyotrophic lateral sclerosis and Wallerian degeneration, where alterations in axonal excitability were greatest during disease stages associated with the largest number of axonal loss (Kanai *et al.*, 2006; Moldovan *et al.*, 2009). As such, these findings would support

the capacity for the ongoing existence of a set of robust and enduring motor neurons, likely related to sufficient SMN protein to provide physiological function. Altogether, axonal excitability measures in the surviving axons from the present study may provide insight into the hypothesis that the rate of neurodegeneration appears to slow reaching a plateau over time, reflecting the capacity for surviving axonal remodelling and collateral sprouting, concurrent with the process of neurodegeneration. Further these mechanisms may promote survival in less severe or chronic forms of spinal muscular atrophy.

Although there remains no effective disease modifying treatment for patients with spinal muscular atrophy, several therapeutic strategies targeting the unique genomic and molecular basis of the disease and neuroprotective approaches are currently under development. Riluzole, the only disease modifying treatment for amyotrophic lateral sclerosis, blocks persistent Na^+ channels (Urbani and Belluzzi, 2000; Kuo *et al.*, 2005; Cheah *et al.*, 2010) and is being explored as a potential therapy in spinal muscular atrophy following an open label study (Russman *et al.*, 2003). Of further relevance, open label and pilot studies in patients with spinal muscular atrophy have suggested potential benefit with valproic acid (Weihl *et al.*, 2006; Swoboda *et al.*, 2009). In addition to its action as a histone deacetylase inhibitor increasing full-length SMN levels, valproic acid blocks voltage-gated Na^+ channels and consequently may also provide neuroprotection in spinal muscular atrophy. As such, results from the present study support further pursuit of these approaches. Clinical trials have held limited success to date, highlighting challenges in design, including utilizing sensitive and relevant outcome measures and targeting a patient group where there is the potential for improvement. As such, findings from the present series suggest that axonal excitability studies may be developed as a marker of spinal muscular atrophy severity and potentially incorporated into future treatment approaches.

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