In vivo evidence for reduced ion channel expression in motor axons of patients with amyotrophic lateral sclerosis

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Abbreviations: ADM, abductor digiti minimi; **ALS**, amyotrophic lateral sclerosis; **APB**, abductor pollicis brevis; **CMAP**, compound muscle action potential; **NC**, normal controls; **RC(2-1)**, difference between subexcitability measured following double and single supramaximal stimuli; **RMP**, resting membrane potential; **S2 accommodation(%)**, reflects the accommodating 'sag' to depolarization and is the difference in excitability at the peak and 90-100 ms after the onset of depolarization.

Abstract

Amyotrophic lateral sclerosis (ALS) is characterised by a progressive loss of motor units and the reinnervation of denervated muscle fibres by surviving motor axons. This reinnervation preserves muscle function until symptom onset, when some 60-80% of motor units have been lost.

We have studied the changes in surviving motor neurons by comparing the nerve excitability properties of 31 single motor axons from patients with ALS with those from 21 single motor axons in control subjects.

ALS motor axons were classified as coming from moderately or severely affected muscles according to the compound muscle action potential amplitude of the parent muscle. Compared with control units, thresholds were increased, and there was reduced inward and outward rectification and greater superexcitability following a conditioning impulse. These abnormalities were greater in axons from severely affected muscles, and were correlated with loss of fine motor skills.

A mathematical model indicated that 99.1% of the differences between the moderately affected ALS and control units could be explained by a reduction in the expression of *all* ion channels. For the severely affected units, modelling required, in addition, an increase in the current leak through and under the myelin sheath. This might be expected if the anchoring proteins responsible for the paranodal seal were reduced.

We conclude that changes in axonal excitability identified in ALS patients are best explained by a failure in the supply of ion channel and other membrane proteins from the diseased motor neuron, a conclusion consistent with recent animal and *in vitro* human data.

Introduction

Amyotrophic lateral sclerosis (ALS) is a heterogeneous disease with the site of symptom onset and the rate of disease progression varying widely between individuals. In 'classical' ALS both upper and lower motor neurons are affected. Additionally there are two dominant hypotheses as to whether the disease process follows a dying-forward or dying-back course, but in either case lower motor neurons are progressively lost from affected motor pools (Eisen & Weber, 2001; Dadon-Nachum *et al.*, 2011; Kiernan *et al.*, 2011; Simon *et al.*, 2014; Vucic *et al.*, 2014; Maglemose *et al.*, 2017). However, denervated muscle fibres attract reinnervation by the surviving motor axons, so that motor units become larger as they become fewer. Indeed, the mechanism of compensation by reinnervation of denervated muscle fibres is so effective that it has been estimated that up to 80% of motor units are lost before a deficit becomes apparent clinically (McComas *et al.*, 1971; Hansen & Ballantyne, 1978; Daube, 2000; de Carvalho & Swash, 2016). This process of denervation and reinnervation may be expected to increase the metabolic demands on surviving neurons as the motor pool becomes more reliant on rate coding as a means of grading force (Daube, 2000; de Carvalho *et al.*, 2014).

Several mechanisms could disrupt the supply of essential proteins to the membrane of the distal axon. Cytoplasmic inclusions in axons are a pathological hallmark of ALS, and provide strong evidence for dysfunction of protein homeostasis (Kabashi & Durham, 2006; Bilsland *et al.*, 2010; Sundaramoorthy *et al.*, 2015; Webster *et al.*, 2017; Brenner *et al.*, 2018).

Deficits of axonal transport have been documented in mouse models of neurodegenerative disease including ALS (Millecamps & Julien, 2013; Brady & Morfini, 2017; De Vos & Hafezparast, 2017), and transport abnormalities have been identified *in vitro* in cell culture lines from familial ALS carrying C-terminal KIF5A splice site mutations (Brenner *et al.*, 2018). Furthermore, post-mortem observations of the accumulation of phosphorylated neurofilaments in ALS indicate a role for axonal transport in

the pathogenesis of ALS (Xiao *et al.*, 2006; De Vos & Hafezparast, 2017). Such changes in the production and maintenance of axonal proteins would be expected to affect the excitability of the distal motor axon.

Non-invasive axonal excitability studies have been used to study the mechanisms underlying pathophysiology in patients *in vivo* with various neuromuscular and neurodegenerative conditions including ALS (Vucic & Kiernan, 2006; Krishnan *et al.*, 2009; Park *et al.*, 2017). Using axonal excitability, an early study found evidence for a reduction in K^+ conductances in patients with ALS, and this was reproduced in the rat *in vitro* using the K^+ channel blockers, tetraethylammonium and 4-aminopyridine (Bostock *et al.*, 1995). Several studies have reported prolongation of the strength-duration time constant, a finding attributed to an up-regulation of persistent-Na^+ currents (Mogyoros *et al.*, 1998; Kanai *et al.*, 2006; Tamura *et al.*, 2006; Vucic & Kiernan, 2006, 2010; Cheah *et al.*, 2012). Furthermore, Kanai *et al.* (2012) reported that a prolonged strength-duration time constant was strongly associated with a shorter survival. The changes in axonal excitability evident in ALS were probably related to disease stage with the earliest changes responsible for increases in persistent-Na^+ currents, and then subsequent loss of K^+ currents (Kanai *et al.*, 2006). These studies put a focus on axonal ion channels as a source of abnormality, clinical features and potentially novel therapies (Park *et al.*, 2015; Park *et al.*, 2017).

These previous studies of nerve excitability changes in ALS have been restricted to recordings of compound action potentials, making it difficult to relate them to changes at the level of the individual motor neuron, since the disease can alter both axonal threshold and motor unit size. The present study was undertaken to avoid this difficulty by studying single motor axons.

It will be argued that, while there are distinctive ion channel abnormalities in ALS axons, they are likely to result principally from a deficit in the supply and maintenance of ion channel proteins to the distal axon.

Materials and Methods

Ethical approval

Patients with a confirmed diagnosis of ALS (Awaji definite) were recruited prospectively from the NHMRC Sydney Health Partners ForeFront Motor Neurone Disease Clinic at the University of Sydney. Those with other conditions that could affect peripheral nerve function were excluded. Patients provided informed written consent prior to commencement of studies. Data for 31 single motor units from 21 ALS patients (17 male, 4 female) with ALS were compared with our data for 21 single motor units from 13 healthy age-matched controls (see Results), 17 from Trevillion *et al.* (2010). Ethical approval was obtained by the Human Research Ethics committee of the University of Sydney (#2014/056, 1st April, 2014) and the studies conformed to the requirements of the Declaration of Helsinki (except for registration in a database).

Clinical assessment

All patients underwent a neurological assessment with formal testing of muscle power. Muscle strength was assessed using the MRC score (Medical Research Council, 1976). Thumb and finger abduction were used to test the abductor pollicis brevis (APB) and abductor digiti minimi (ADM) muscles, respectively. All patients with or without wasting had weakness of the tested hand.

The revised ALS functional rating scale (ALSFRS) was used as an overall measure of disease burden and measured out of 48, with lower scores denoting higher disease burden (Cedarbaum *et al.*, 1999). Components that rely on hand function (the *Handwriting*, *Feeding* and *Dressing and hygiene* sections) were combined to provide a fine motor ALSFRS sub-score out of 12. The progression rate was calculated using the following formula [48 – ALSFRS] / [Disease duration in months] (Labra *et al.*, 2015).

ALS motor units were considered to come from moderately and severely affected muscles on the basis of the size of the compound muscle action potential (CMAP) of the parent muscle, > 1 mV and < 1mV, respectively, in accordance with Kanai *et al.* (2006).

Electrophysiology

Recording setup

Motor unit recordings were obtained from the APB and ADM muscles of ALS patients. Disposable Ag/AgCl ECG-type electrodes (WhiteSensor WS; Ambu A/S, Ballerup, Denmark) were secured to the skin over the muscle belly and on the corresponding proximal phalanx. The same type of electrode was used on the dorsum of the hand for signal ground. Wet-gel Ag/AgCl electrodes (WhiteSensor 4500M-H; Ambu A/S, Ballerup, Denmark) were used for stimulation. The optimal location for the cathode was sought with a cloth-covered Ag/AgCl searching electrode and the anode was sited approximately 10 cm proximal and off the nerve under study. Skin temperature was monitored continuously throughout each experiment adjacent to the stimulus site at the wrist, and was kept at >32°C (Burke *et al.*, 1999; Kiernan *et al.*, 2001a).

Single motor unit action potentials and compound muscle action potentials were amplified using a purpose-built low-noise amplifier (Howells *et al.*, 2018; gain x250; filter 2 Hz - 2 kHz) before having mains frequency noise removed using a 50-Hz noise eliminator (Humbug noise eliminator; Quest Scientific, Vancouver, Canada). Isolation of single motor units is difficult in healthy subjects using Ag/AgCl surface electrodes, and the 17 units from Trevillion *et al.* (2010) were recorded using a double differential recording electrode with an active sensor (DE3.1, Delsys Inc., Natick, Massachusetts, USA). The additional four control units were isolated (with difficulty) using the same technique as in patients. These waveforms were sampled at 10 kHz by a data acquisition system (NIDAQ USB-6221) which was under the control of a recording script within the QtracS software (TRONDNF.QRP; © ION, UCL, UK). This recording script also provided the command waveforms for control of a bipolar constant-current stimulator (DS5; Digitimer Ltd, Welwyn Garden City, UK).

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An estimate of the number of motor units was made using a CMAP scan and the *MSCANFit* method (Bostock, 2016; Farschtschi *et al.*, 2017; Jacobsen *et al.*, 2017). The CMAP scan is a finely graded stimulus response curve, which plots the size of the compound muscle action potential in response to stimuli of decreasing intensity. The 200-μs stimuli began at a supramaximal intensity and each subsequent stimulus was reduced by 0.2% until no response could be detected. The *MSCANFit* procedure is a statistical method which estimates the number of motor units by assessing the size and variability in recruitment threshold of all of the motor units contributing to the compound response (Bostock, 2016).

Axonal excitability

Conventional axonal excitability recordings track changes in the stimulus required to produce a fixed fraction of the entire motor pool under study. The present study however focussed on the properties of individual motor units. The threshold of the single motor unit was continually tracked by adjusting the 1-ms stimulus to produce an all-or-none response. Single units could be used only if their threshold was clearly distinguishable from those of other units. For threshold-tracking purposes clear discrimination of a single unit was obtained when the next-recruited units had thresholds that were substantially higher, or they were obviously smaller or of a different latency than the unit under study (Fig. 1). It was generally easier to discriminate the low-threshold units in ALS subjects than in control subjects, because there were fewer units in ALS and those units were relatively large.

Threshold related measures

The stimulus required to elicit the all-or-none response of the single motor unit was tracked throughout the entire excitability recording and provided the control threshold for calculation of threshold changes and for setting conditioning stimulus intensities.

Strength-duration properties were recorded by measuring the unit threshold using rectangular pulses of different duration (0.2, 0.4, 0.6, 0.8 and 1ms). The strength-duration time constant and rheobase were calculated using Weiss's law plotting the stimulus charge versus stimulus duration (Weiss, 1901; Bostock, 1983; Mogyoros *et al.*, 1996).

Threshold electrotonus and the current-threshold relationship

Threshold electrotonus measures the excitability before, during and after 100-ms long subthreshold conditioning currents. The strength of the conditioning currents were set to ±20% and ±40% of the control threshold. The depolarizing currents (+20, +40%) probe the delayed outward rectification attributed to fast and slow K^+ channels of the K_v1 and K_v7 families, respectively. The hyperpolarizing currents probe the inwardly rectifying current (I_h) which passes through hyperpolarization activated cyclic nucleotide gated (HCN) channels. The accommodation to depolarization responsible for the 'sag' in excitability of depolarizing threshold electrotonus is primarily due to outward rectifying slow K^+ channels and is denoted here as *S2 accommodation*. *TEd(90-100ms)* and *TEh(90-100ms)* represent the threshold reduction at the end of the 40% depolarizing and -40% hyperpolarizing conditioning currents and are sensitive to changes in membrane potential (Kiernan & Bostock, 2000).

The current-threshold relationship is the threshold-tracking analogue of traditional current/voltage measurements from ion channel electrophysiology (Kiernan *et al.*, 2000; Kiernan *et al.*, 2001b). The threshold for activation of a single motor unit was tested at the end of 200-ms long conditioning currents which varied in strength from +50% (depolarizing) to -100% (hyperpolarizing) of the control threshold. The slope of the curve reflects accommodation to the current injection, the steeper the curve with depolarizing currents the greater the accommodation due to K^+ currents, and the steeper the curve with hyperpolarizing currents the greater the accommodation due to I_h. The slope of the current-threshold relationship as the current passes from hyperpolarization to depolarization is denoted as *resting IV slope* and is the threshold analogue of resting input conductance.

Recovery of excitability following the discharge of a single motor unit

Following the discharge of a single motor unit, there is a characteristic pattern of excitability changes known as the recovery cycle, with phases of refractoriness, superexcitability and late subexcitability. Immediately following a discharge the axon is absolutely refractory (inexcitable), then relatively refractory where the threshold is greater than the control threshold. Refractoriness gives way to a period of superexcitability where the threshold is lower than the control threshold, and then a period of late subexcitability where again the threshold is greater than the control. These fluctuations in excitability are generally complete within 200 ms. To characterise better the activity of slow K^+ channels, the recovery of excitability following a double suprathreshold conditioning pulse was also recorded (Shibuta *et al.*, 2010b). From this, a measure that accurately reflects slow K^+ activity (Shibuta *et al.*, 2010b) was calculated as the difference between the subexcitability following the double pulse and the subexcitability following a single pulse. This measure is denoted here as *RC(2-1)*. The period of subexcitability following activation typically occurs some 30 to 100ms after the conditioning pulse(s).

Abnormal Excitability Index

To quantify the extent of abnormality of the ALS single motor units an Abnormal Excitability Index (AEI) was developed. The index reflects the key differences in excitability parameters in the present study between ALS and control units: *resting IV slope*, *TEh(90-100ms,%)*, *TEd(90-100ms,%)* and *superexcitability* :

$$AEI = \frac{Z_{TEd(90-100ms,\%)} - Z_{restingIVslope} - Z_{TEh(90-100ms,\%)} - Z_{superexcitability}}{4}$$

Where $Z_{restingIVslope} = \frac{restingIVslope_{ALS} - \mu_{restingIVslope_{NC}}}{\sigma_{restingIVslope_{NC}}}$ and

$\mu_{restingIVslope_{NC}}, \sigma_{restingIVslope_{NC}}$ are the mean and standard deviation of the *resting IV slope* values for the control subjects, respectively.

$Z_{TEh(90-100ms,\%)}, Z_{superexcitability}, \quad Z_{TEd(90-100ms,\%)}$ were calculated in a similar manner.

The AEI was constructed such that membrane hyperpolarization or depolarization produced positive or negative AEIs, respectively. Essentially the AEI represents the average number of standard deviations each of these key measures is away from the mean of the control data.

Mathematical modelling

The biophysical basis for the underlying changes in ALS motor units was explored using the 'Bostock' model of a human motor axon as implemented in the QtracP software (Kiernan *et al.*, 2005; Bostock, 2006). A complete description of the model is presented in Howells *et al.* (2012). Briefly, the model consists of a node and internode which are coupled by the 'Barrett-Barrett' pathways through and under the myelin sheath (Barrett & Barrett, 1982). Threshold-tracking studies probe the excitability of the nodal membrane as influenced by the internode, incorporating the properties of key ion channels, pumps and their interconnections. Transient and persistent Na^+ currents are modelled at the node, while slow and fast K^+ currents are included at both the node and internode, and the inwardly-rectifying current, I_h is internodally located. Na^+/K^+-ATPase pump and 'leak' currents are also represented at both the node and internode.

The model was first fitted to the control single motor unit data to create a normal control model and then changes from the normal control model were explored by minimising the discrepancy between the model and the ALS single motor unit data.

Statistical analysis

Normality was assessed using the Shapiro-Wilk test. For normally distributed data, group data are presented as the mean ± standard error of the mean. Data that followed a log-normal distribution are presented as the geometric mean ×/÷ geometric standard error of the mean (expressed as a factor). The remaining non-parametric data are presented as the median {interquartile range, IQR}.

Differences between the data for the control and patient groups (moderately and severely affected) were assessed using a one-way ANOVA. Specifically, parametric data were analysed using an ANOVA

with Dunnett's and Tukey multiple comparisons tests, and the non-parametric data with the Kruskal-Wallis and Dunn's post-hoc tests.

The MRC, ALS, ALSFRS-fine scales are ordinal and as such were correlated with axonal excitability data using Spearman rank-order analysis.

Results

All 21 patients had an Awaji-definite diagnosis of ALS (de Carvalho *et al.*, 2008). The mean disease duration from symptom onset (25.9 ×/÷ 1.1 months) and clinical phenotypes were consistent with a representative ALS cohort (upper-limb onset, 62%; lower-limb onset, 14%; bulbar onset, 24%) (Kiernan *et al.*, 2011). At the time of measurement 95% of patients were receiving riluzole, the effects of which on nerve excitability have been documented elsewhere (Vucic *et al.*, 2013).

Recordings from 31 single motor units were made from APB (48%) and ADM (52%), to allow insights into potential differences related to the "split-hand syndrome" in ALS, a pathognomonic feature of ALS (Wilbourn, 2000; Eisen & Kuwabara, 2012; Menon *et al.*, 2013; Menon *et al.*, 2014). The excitability of ALS single motor units was compared to 21 single motor units from age-matched healthy controls (ALS, median age 54 years old [IQR: 44-65]; NC (median) 40 [IQR: 35-63.5]; p=0.13, Mann-Whitney U-test). There was no significant difference in the temperature between ALS and control recordings (ALS, 33.8 ± 0.2°C; NC, 34.2 ± 0.2°C; p=0.24).

The ALS single motor units were obtained from a muscle that had undergone significant atrophy when compared to healthy controls, as shown in Fig. 2A (maximal CMAP: NC 8.8 mV ×/÷ 1.1; moderate ALS, 2.5 mV ×/÷ 1.1, p=3.8 × 10^{-10}; severe ALS, 0.5 mV ×/÷ 1.3, p=2.2 × 10^{-9};). This was also reflected in the number of surviving motor units in each muscle studied with an estimate obtained using the CMAP scan, of 12.6 ± 1.7 and 2.7 ± 0.8 motor units in the moderately and severely affected ALS patients groups, respectively (range 1 – 33) compared to normative data for this technique (greater than 70 units; Farschtschi *et al.*, 2017). The average size of motor units was 699 ± 107 μV

(moderately affected ALS) and 639 ± 193 µV (severely affected ALS). As noted in Methods, only four healthy control motor units were recorded using the same electrode montage as for the ALS units, and these were smaller than in ALS (30, 31, 50 and 220 µV). These findings indicate a substantial loss of motor units with larger surviving units, presumably due to reinnervation of denervated muscle fibres.

Axonal function

Recordings for single motor units were usually complete within 30 minutes, and were more readily achievable in ALS patients due to the presence of fewer (but larger) motor units, whose thresholds differed markedly from those of any adjacent units in the CMAP scan, making them easier to isolate than units in healthy controls. A typical threshold tracking recording for an ALS single motor unit is shown in Figure 1. The 'all-or-none' response is clearly evident in Fig. 1A, and the unconditioned and conditioned stimulus thresholds for activation are shown as black and coloured lines, respectively (Fig. 1B). Some motor axons were excited by the 40% of threshold conditioning current used for depolarizing threshold electrotonus, something that does not normally happen with motor recordings from healthy nerve (Trevillion *et al.*, 2007), suggesting axonal hyperexcitability.

Differences across the ALS 'split hand'

Across the ALS cohort, there was a trend for a greater number of motor units in ADM than APB motor pools when assessed using the CMAP scan (APB, 4.1 ×/÷ 1.3; ADM, 8.3 ×/÷ 1.3; p=0.06). Similarly, the maximal CMAP was larger in ADM than APB, though this was also not significant (APB, 1.1 mV ×/÷ 1.3; ADM 1.7 mV ×/÷ 1.3; p=0.3). As such, four of the 16 ADM units and six of the 15 motor units were allocated to the 'severely' affected' group. These trends are consistent with the ALS 'split hand' phenomenon, with preferential wasting of thenar muscles when compared to hypothenar muscles (Wilbourn, 2000; Eisen & Kuwabara, 2012; Menon *et al.*, 2013). However, there were no significant differences in threshold, rheobase or strength-duration time constant of motor units recorded in the APB and ADM muscles. In addition, there were no significant differences in

other axonal excitability measures between the APB and ADM muscles (Fig. 3). These findings are consistent with an earlier report on the absence of systematic differences in axonal excitability for different intrinsic muscles of the hand in studies using compound potentials (Menon *et al.*, 2014).

The motor units of APB and ADM were therefore combined in subsequent analyses.

Threshold related measures

Rheobase and threshold for activation of single motor units were significantly increased in ALS when compared to controls (*p*=0.002 and *p*=0.001, respectively; Figs 2, 4). In contrast to earlier studies that used compound targets (Mogyoros *et al.*, 1998; Kanai *et al.*, 2006; Tamura *et al.*, 2006; Vucic & Kiernan, 2006, 2010; Cheah *et al.*, 2012), the Strength-Duration Time Constant was not longer in the single motor units of ALS subjects than controls (*p*=0.8; Figs 2, 4 and Table 1).

Consistent with previous findings rheobase was inversely correlated with the strength-duration properties for the control motor units. The relationship for the moderately affected ALS group was not significant, possibly reflecting a more heterogeneous population of motor units (Fig. 4A; see Mogyoros *et al.*, 1998). This will be addressed in the Discussion.

Polarization of the nodal and internodal membranes

The response to long-lasting polarization is best observed during threshold electrotonus and current-threshold measurements (Fig. 5A; Table 1), and they identified a reduction in outwardly rectifying conductances in the ALS units, more so for the more severely affected group (compare upper-most blue with green and red traces in Fig. 5A). This finding is supported by significantly greater threshold reductions across a number of measures in depolarizing threshold electrotonus: the peak of depolarizing threshold electrotonus, *TEd(peak,%)*; 40 to 60ms after the onset of depolarization, *TEd(40-60ms,%)*; and at the end of the 100-ms depolarization, *TEd(90-100ms,%)*. Similarly, in the current-threshold relationship there was a greater threshold change to depolarizing currents (see top right quadrant in Fig. 5C). With depolarizing currents +50% of the control threshold, the

threshold change was 56.0 ± 1.7% and 59.7 ± 2.2% for the moderate and severe ALS groups, and 48.4% ± 1.0 (p=3.8 x 10^{-5}) in control units.

Similarly, inward rectification was reduced in the ALS units, with significant threshold increases in response to hyperpolarization evident during threshold electrotonus, *TEh(90-100ms,%)*; and in the current-threshold relationship (see lower left quadrant in Fig. 5C). Hyperpolarizing currents -100% of control threshold produced threshold changes of -308.5 ± 15.3% and -339.8 ± 37.6% in the moderate and severe ALS groups, and -243 += 9.7% in control units (p=0.001). The slope of the current-threshold plot is plotted against threshold reduction in Fig. 5D for each group. This is the threshold analogue of a conductance versus membrane potential plot. It revealed a progressive reduction in minimum and resting conductances (minimum and resting IV slopes, respectively) and at all levels of polarization (from +50% depolarization through to -100% hyperpolarization), suggesting a reduction in multiple voltage-dependent conductances across the range of membrane potentials studied.

Recovery cycle

Finally, the recovery of excitability following the discharge of a single motor unit was markedly different between ALS patients and controls with a significantly shorter relative refractory period and greater superexcitability in ALS (Fig. 5B and Table 1).

Abnormal Excitability Index

Taken together, the pattern of change of excitability across single motor units in ALS resembled a pattern that can be induced by hyperpolarization of membrane potential (Kiernan & Bostock, 2000), with increased thresholds, "fanning out" of threshold electrotonus, reduced inward and outward rectification in the current-threshold relationship and an increase in superexcitability following passage of an action potential. Indeed 87% of the ALS single motor unit recordings exhibited the "fanned out" pattern of excitability, and the mean Abnormal Excitability Index for all groups was significantly different from each other with the moderately and severely affected axons progressively more abnormal than the normal control group (AEI: NC, 0.0 ± 0.2; moderate, 1.1 ± 0.2;

severe, 2.5 ± 0.6. ANOVA: NC vs moderate, p=0.0056; NC vs severe, p<0.0001; moderate vs severe, p=0.0052; Fig. 6A). The most positive values of Abnormal Excitability Index represent the most abnormal recordings which were from the more affected muscles.

The Abnormal Excitability Index was negatively correlated with specific measures of hand function, such as the number of motor units, MRC score and ALS fine motor subscore (Table 2). Specifically, the more abnormal the excitability index, the greater the loss of fine motor skills (Fig. 6B, C). However the Abnormal Excitability Index was not correlated with maximal CMAP size, the amplitude or threshold of the single motor unit studied. Similarly, it was not correlated with disease duration, total ALSFRS or progression rate.

Within-subject comparisons

In six of the ALS subjects multiple recordings were made in different muscles. Specifically, in four subjects recordings from units in the APB and ADM muscles were made ipsilaterally. In another subject recordings from both APB and ADM were made bilaterally, and in the final subject recordings from ADM were made bilaterally (see Table 3).

There were no significant differences in the excitability variables or the Abnormal Excitability Index for the APB and ADM muscles (n=6) in the same subjects. Three APB and ADM muscle pairs had the same muscle strength as assessed by MRC; neither of these pairs nor the remaining pairs with different strengths demonstrated significant differences in the excitability variables or the Abnormal Excitability Index. Similarly, no significant differences were evident when the APB/ADM muscle pairs were assessed with similar and different ALSFRS fine motor sub-scores though, for each comparison, the number of unit pairs was small.

Insights from mathematical modelling

Differences in the excitability of single motor units in ALS patients were explored using a mathematical model of the behaviour of human motor axons. The model was first fitted to the control single motor unit data (red filled circles in Fig. 7) to create a normal control model (red lines

in Fig. 7). The changes from the normal control model necessary to reproduce the ALS recordings were then used to explore the underlying mechanisms of the abnormal ALS single motor units.

Given the overall pattern of changes we started with whether membrane hyperpolarization could reproduce the differences between ALS and control units. The best reduction in discrepancy between ALS single motor unit data and controls was 37.3% (moderate) and 29.2% (severe), and this was achieved by hyperpolarization of RMP (from -81.6 to -83.7 and -84.2 mV). This suggests that hyperpolarization cannot explain the ALS data.

Previous studies have suggested abnormal expression of axonal membrane ion channels, and accordingly each of these conductances was explored individually. Variation of the expression of Na^+ channels on the nodal membrane was unable to reduce the discrepancy between the normal control model and ALS motor units. Variation of the percentage of persistent Na^+ channels alone did not account for any of the changes recorded in ALS patients. A reduction in K^+ channel expression alone had a minor effect on the discrepancy between ALS motor units and controls. The maximal improvement in the fit produced by reducing slow or fast K^+ channel activity was 5.5% (though when both slow and fast K^+ were decreased together the maximal improvement was 19.5%). Taken together these, findings indicate that alterations in only Na^+ or only K^+ channel function cannot explain the observed changes in ALS.

An alternative mechanism is that diseased motor neurons might be incapable of supplying sufficient membrane proteins to the distal axon. Therefore, the expression of all modelled conductances were allowed to vary together. Unexpectedly, an equal reduction of Na^+, slow and fast K^+, HCN and 'leak' channels by 28.9% and 44.3% accounted for 96.1% and 92.5% of the discrepancy between the moderate and severe groups and the normal control model. However it is unlikely that each channel would be affected to the same degree (see Discussion), and when the expression of all modelled conductances and the pump current were allowed to vary independently, the discrepancy between normal control model and ALS data was reduced by 99.1% (moderate) and 98.5% (severe). The

modelling results are shown in Figure 7, where the filled circles represent the mean data and the lines represent the best-fit outputs for the mathematical model. The model was able to demonstrate the progressive abnormality in ALS axons (normal to moderate to severe, shown as red, green and blue, respectively) - increased 'fanning out' of threshold electrotonus (Fig. 7A), increased superexcitability (Fig. 7B), and decreased inward and outward rectification (lower left in Fig. 7C,D and upper right in Fig. 7C,D, respectively). Importantly, a reduction in all channel conductances, apart from the Barrett-Barrett pathway under the myelin sheath, contributed to the observed abnormal excitability in the ALS patients (moderate group: Na^+, 24%; slow K^+, 17%; fast K^+, 26%; 'leak' channels, 35%; HCN channels, 32%; severe group: Na^+, 22%; slow K^+, 35%; fast K^+, 35%; 'leak' channels, 43%; HCN channels, 34%). In contrast to the reductions above there was an increase (16%) in the Barrett-Barrett conductance, but this occurred only in the severe group. An increase in the Barrett-Barrett conductance can be explained if there is a reduction in expression of axoglial proteins that anchor the myelin sheath to the axonal membrane. There were slight increases in the pump current (moderate, 13.3 pA; severe, 7.7 pA), but this was insufficient to offset the depolarizing effect of reducing K^+ expression and the overall effect on resting membrane potential was a *depolarization* of 0.4 mV (moderate) and 0.8 mV (severe).

Reinnervation and the activity of individual ion channels.

ALS is characterised not only by axonal degeneration, but also by reinnervation of denervated muscle fibres by surviving axons. On the assumption that the size of the individual motor unit is an indication of the extent of sprouting and reinnervation of denervated muscle fibres, correlations were sought with measures related to individual ion channels. There were no correlations of unit size with measures dependent on Na^+, fast K^+, or I_h channels. However, there was a clear correlation between unit size and measures of slow K^+ activity. *S2 accommodation* and *RC(2-1)* are the most specific axonal excitability measures of slow K^+ function, and were strongly correlated in the ALS single units (Fig. 8A). Both *S2 accommodation* and *RC(2-1)* were positively correlated with the logarithm of unit size (Fig. 8B,C), suggesting greater slow K^+ activity with increasing unit size.

Discussion

The present study has established the behaviour and functional properties of single motor axons in patients diagnosed with ALS. The markedly abnormal alterations in excitability and thereby function cannot be attributed to relatively normal axons from small surviving motor neurons (Shibuta *et al.*, 2010a; Trevillion *et al.*, 2010). These changes can however be explained by a failure of supply to the peripheral axon of channel and other membrane proteins from a diseased motor neuron. It is relevant that, in motor neurons derived from human induced pluripotent stem cells harbouring TARDBP and C9orf72 ALS mutations, there is a progressive decrease in Na^+ and K^+ currents (Devlin *et al.*, 2015). A unique aspect of the present study is the evidence for such a defect in ALS from *in vivo* studies. The Barrett-Barrett conductance (while increased) is effectively a leakage conductance, dependent on the integrity of the paranodal seal and the supply of adhesion molecules. As such a reduction in the supply of proteins to the axonal membrane can account for the abnormalities identified in the present study.

Axonal excitability and ion channel homeostasis.

Several mechanisms of ALS neurodegeneration could limit the turnover of ion channels and associated proteins on the membrane of the distal motor axon. Synthesis of ready-made transmembrane proteins, packaging for transport, axonal transport, local synthesis, insertion and failure of protein quality control into the membrane could all be affected in ALS. Evidence for limited protein turnover may be seen in the cytoplasmic inclusions in upper and lower motor neurons that are a neuropathological hallmark of ALS (Al-Chalabi *et al.*, 2012; Blokhuis *et al.*, 2013).

Decreased axoplasmic transport has been documented in mouse models of ALS and other neurodegenerative diseases (Bilsland *et al.*, 2010; Millecamps & Julien, 2013; Brady & Morfini, 2017; De Vos & Hafezparast, 2017), and defects in intracellular transport have been identified *in vitro* in familial ALS (Brenner *et al.*, 2018).

Local mechanisms have also been implicated in ALS, with a failure of protein quality control, local synthesis and insertion all impacting on protein homeostasis (Kabashi & Durham, 2006; Sundaramoorthy et al., 2015; Cestra et al., 2017; Webster et al., 2017).

Reinnervation and axonal sprouting

We had supposed that the extra membrane requirements for the expansion of surviving motor units in ALS might be a significant factor in the loss of ion channel function, but there was no relationship between the abnormal excitability index and motor unit size. Membrane dysfunction was related to measures of fine motor control, but not to the expansion of the particular motor unit. However, measures of slow K^+ function were correlated with the size of individual motor units. It is conceivable that this is a secondary phenomenon, with the larger ALS motor units compensating for the spontaneous activity of immature terminal sprouts by attempting to upregulate slow K^+ currents. There are previous studies of axonal excitability in regenerating nerve by Moldovan and Krarup (2004b, 2004a) and (Moldovan et al., 2016), who presented evidence for axonal hyperpolarization which they attributed to increased activity of the electrogenic Na^+/K^+ pump, to maintain Na^+ balance in the regenerating axons. We found no such evidence in ALS axons, and the critical difference is likely to be that their studies involved the properties of actively regrowing axons, not the properties of intact axons that have sprouted distally to reinnervate denervated muscle fibres.

Why was there no difference in the Strength-Duration Time Constant?

The absence of a change in the strength-duration time constant in the present study contrasts with the findings in earlier reports (Mogyoros et al., 1998; Kanai et al., 2006; Tamura et al., 2006; Vucic & Kiernan, 2006, 2010; Cheah et al., 2012). There is likely to be no single explanation for this discrepancy, but a number of issues need to be considered. First, the CMAP studies looked at a population of motor units, not just one, and there is likely to be heterogeneity in the properties of the pool. Secondly, the changes in the strength-duration time constant were difficult to demonstrate in some previous studies (e.g., Mogyoros et al., 1998; Tamura et al., 2006), and was reported to be

significant only for patients with a preserved CMAP (Kanai *et al.*, 2006). Thirdly, all patients in this study were on riluzole, and this is a Na^+ channel blocker, preferentially affecting their persistent behaviour (Xie *et al.*, 2011). Finally, the strength-duration time constant depends on the balance between persistent Na^+, K^+, leak conductances and passive membrane properties. It is conceivable that the turnover of K^+ channels is faster than that of Na^+ channels, so that initially only K^+ channels are reduced, resulting in depolarization and an increase in the strength-duration time constant. This may provide further insight into the findings of Kanai *et al.* (2012) who found that the higher strength-duration time constants correlated with shorter survival in ALS patients. It is reasonable to presume that more rapidly degenerating units would have a greater impairment of axonal supply mechanisms (such as protein synthesis, axonal transport etc.) and therefore a greater imbalance in axonal ion channels underlying the strength-duration time constant.

This study provides no simple explanation for hyperexcitability responsible for fasciculations. Indeed it provides evidence *against* a favoured hypothesis that persistent Na^+ currents are increased. However, a simple decrease in K^+ currents would be expected to produce increased excitability. We note however that the ectopic activity causing fasciculations occurs mainly in the motor axon terminals and sometimes proximally near the cell body (Roth, 1982; de Carvalho & Swash, 2013), not mid-axon (Brigant & Mallart, 1982; Mallart, 1985), but we have no insights into how they might change to produce the hyperexcitability.

Clinical correlations

While the patients had a split-hand syndrome clinically (and there was some neurophysiological support for this), motor axons innervating the thenar and hypothenar muscles did not differ significantly and there was no correlation between the abnormality in axonal excitability and the rate of disease progression.

Importantly however there were strong correlations with fine motor skills for the involved hand, but only when all of the motor unit data were included in the analysis. This will be discussed below.

The "Abnormal Excitability Index" may represent a useful index for following the progression of disease within individual motor units.

The correlation with severity is consistent with a progressive dysfunction of the surviving motor neurons, and confirms the value of axonal excitability studies in following disease progress and any response to therapy (Turner *et al.*, 2009).

Heterogeneity of moderately affected ALS motor units

There are several reasons why correlations between the commonly used clinical variables and axonal excitability parameters were not evident in the moderately-affected ALS single motor units. First, these widely used clinical parameters may not accurately reflect the state of the motor pool: maximal CMAP is confounded by the reinnervation of denervated muscle fibres and will not accurately reflect the number of motoneurons, while MRC and ALSFRS-fine are affected by loss of descending drive from upper motor neurons. Perhaps the cleanest functional measure of the state of the lower motor neuron pool, is the *motor unit number estimate*. Secondly, it is likely that the moderately-affected ALS group have a heterogeneous population of single motor units, some relatively normal, some undergoing degenerative changes. This might explain why the expected correlation between strength-duration time constant and rheobase was not evident in the moderate group. Further work needs to be performed to follow individual motor units longitudinally to see how they transition from normal to abnormal excitability. The application of high-density surface EMG using electrode arrays to measure axonal excitability measurements for a number of units should make this task easier to perform (Sleutjes *et al.*, 2018).

Conclusion

A disruption in protein homeostasis is thought to play a critical role in the pathogenesis of ALS, and this study presents the first *in vivo* evidence for a non-selective reduction in ion channel expression in patients. It is not surprising that a diseased motor neuron may have difficulty maintaining a supply of axonal proteins to the membrane surface throughout its length.

Additional information

Author contributions

JH, MCK and DB conceived and designed the study. All authors contributed to critical analysis and interpretation of the data, and drafting of the manuscript. All authors have approved the final draft of this manuscript.

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Table 1.

Excitability differences between single motor units from ALS and control subjects.

Parameter	Normal Control	ALS		ANOVA/{Kruskal Wallis}	
		moderate	severe	p-value	p-value
	(n=21)	(n=20)	(n=11)	NC-moderate	NC-severe
Threshold for single unit (log mA)[*]	2.60 [1.07]	3.70 [1.11]	5.17 [1.22]	0.013	0.0001
Rheobase (log mA)[*]	1.76 [1.09]	2.51 [1.11]	3.48 [1.23]	0.050	0.001
Strength-Duration Time Constant (μs)	482 (32)	493 (26)	431 (26)	0.95	0.44
RRP (log ms)[*]	3.34 [1.05]	2.78 [1.03]	2.51 [1.04]	0.0078	0.0003
Superexcitability (%)	-22.0 (1.8)	-31.3 (2.5)	-42.1 (3.8)	0.013	0.0001
Subexcitability (%)	11.4 (0.8)	10.8 (1.3)	10.1 (1.6)	0.89	0.71
TEd(peak,%)	62.5 (1.8)	68.7 (1.9)	73.5 (3.7)	0.060	0.0055
TEd(40-60ms,%)[†]	46.6 {44.9-50.1}	55.7 {50.4-59.1}	57.5 {54.8-62.8}	{0.0004}	{<0.0001}
TEd(90-100ms,%)[†]	42.0 {39.8-44.9}	48.2 {44.0-50.6}	50.9 {47.2-54.0}	{0.0046}	{0.0002}
TEh(90-100ms,%)	-88.6 (4.1)	-114.6 (4.8)	-144.6 (14.6)	0.0097	0.0001
Resting IV slope	0.667 (0.02)	0.547 (0.02)	0.491 (0.05)	0.0021	0.0002
Minimum IV slope	0.341 (0.018)	0.270 (0.015)	0.251 (0.026)	0.010	0.0056
Hyperpolarizing IV slope[†]	0.396 {0.343-0.439}	0.341 {0.269-0.443}	0.415 {0.332-0.552}	{0.27}	{1}

Data that are normally distributed are presented as mean (standard error of the mean).
[*]Data that are log-normally distributed (threshold, rheobase and RRP) are presented as geometric mean [geometric standard error of the mean].
[†]Non-parametric data (TEd(peak,%), TEd(40-60ms,%), TEd(90-100ms,%), Hyperpolarizing IV slope) are presented as median {interquartile range}.
One-way analysis of variance was performed for the normally distributed data using an ANOVA with Dunnett's post-hoc test. Similarly, analysis of variance was performed for the non-parametric data using the Kruskal-Wallis test with Dunn's post-hoc testing.

Table 2.

Correlation of Axonal Excitability Index with clinical measures.

Clinical measure	Axonal Excitability Index	
	ρ	p-value
MUNE (log number of motor units)	-0.44	0.016
MRC	-0.511	0.0033
ALSFRS-fine	-0.547	0.0019
Maximal CMAP (log mV)	-0.258	0.16
Unit size (log mV)	0.04	0.81
Unit threshold (log mA)	0.235	0.20
Symptom duration (months)	0.132	0.49
ALSFRS	-0.203	0.27
Progression rate	0.103	0.59

Correlation of axonal excitability index using Spearman rank correlation.

Table 3.

Within-subject comparisons of ALS single motor units.

Subject	Muscle	Symptom (months)	ALSFRS (max 48)	ALSFRS-fine (max 12)	MRC (max 5)	CMAP (mV)	AEI
#1	Left APB	17	39	9	3	0.15	0.59
	Left ADM	21	22	2	0	0.09	2.39
#2	Left APB	14	43	10	3	1.38	1.12
	Left ADM	14	43	10	3	0.98	0.55
#3	Left APB	108	36	5	4	5.03	1.32
	Left ADM	108	36	5	2	4.20	1.43
#4	Right APB	8	45	10	4	2.64	2.38
	Right ADM	8	45	10	4	2.04	2.23
#5	Right APB	25	38	4	3	0.86	4.44
	Right ADM	30	36	4	0	0.47	2.78
	Left APB	31	36	4	1	1.92	4.53
	Left ADM	31	36	4	1	0.999	2.55
#6	Left ADM	11	42	8	4	4.88	-0.37
	Right ADM	17	36	4	3	1.92	-0.03

Multiple recordings were made from different muscles in six ALS subjects. In subjects 1-4, recordings from the APB and ADM muscles were made ipsilaterally. In subject #5 both APB and ADM were

recorded bilaterally. In subject #6 single motor units from the ADM muscles were recorded bilaterally.

Figure Descriptions

Figure 1: Threshold-tracking an ALS single motor unit.

A. Traces of single motor unit 'all-or-none' response to small changes in stimulus amplitude above and below the motor unit's threshold. Note the F-wave at 40 ms, of identical shape to the direct response.

B. The black horizontal line represents the unconditioned stimulus threshold for the single unit. The single motor unit recording consists of four parts: **QT**, charge/threshold relationship from which strength-duration time constant can be calculated (the red line in this section represents the stimulus intensity for stimuli of different durations); **TE**, threshold electrotonus (the red, blue, grey and green lines refer to the conditioned thresholds tested at various intervals of current strengths [+40, +20, -20, -40%, respectively]); **IV**, current-threshold relationship (the blue line refers to the stimulus threshold tested at the end of a 200-ms long conditioning current of varying strength (+50 to -100% of the unconditioned threshold); **RC**, recovery cycle (the light blue line refers to the test stimulus and response following activation of a single motor unit at various conditioning-test intervals).

C. The coloured dots represent the response or non-response to the stimuli in **B**. In this example the single unit response had an amplitude of ~0.9 mV. The colours are the same as those in **B** (eg black dots are the responses for the unconditioned stimuli, and the red dots in QT are the responses to stimuli of different durations).

Figure 2: Maximal CMAP and single motor unit threshold-related measures in ALS.

A. The maximal CMAP obtained from stimulation of the same nerve that the single units came from. ALS data were allocated into moderately (green, n=20) and severely affected (blue, n=11) groups on the basis of CMAP size, >1mV and <1mV, respectively. Control data (n=21) are shown in red, and the

CMAPs were significantly greater than the moderate and severely affected groups ($p=3.8 \times 10^{-10}$ and $p=2.2 \times 10^{-9}$, respectively). **B.** Threshold for activation of single motor units was significantly higher in ALS subjects than controls, $p=0.001$. **C.** The rheobase of single motor units was significantly higher in ALS subjects than in controls, $p=0.002$. **D.** There was no significant difference in strength-duration time constant between single motor units in ALS and controls ($p=0.8$), unlike findings in earlier reports, indicating that the changes in rheobasic threshold are those expected for the change in threshold to a 1-ms stimulus, indicating that the changes in rheobasic threshold probably reflect the change in threshold to a 1-ms stimulus shown in **B**.

Figure 3: Axonal excitability of ALS single motor units in ADM and APB.

Solid lines represent the mean, and dashed lines the standard error of the mean. APB units (n=15 units) are shown as filled blue squares and the ADM data (n=16 units) as empty blue squares. **A.** There were no significant differences in any of the parameters derived from the threshold electrotonus recordings of APB and ADM units. **B.** There was a trend toward greater superexcitability in the single motor units measured in APB than ADM ($p=0.055$), but when a multiple comparison correction was applied to the data ($p=0.69$).

C. None of the parameters associated with the current-threshold relationship were significantly different, as can be seen better in **D**.

Figure 4: Relationship of threshold and strength-duration properties.

A. There was a strong inverse correlation of strength-duration time constant and rheobase for single motor units of controls (red), but this relationship was more variable for units from ALS subjects. Dashed ellipse indicates 95% confidence limit of the data and the line of best fit is shown unbroken.

ALS moderately affected (green), Y = 0.534 − 0.014*X, R=-0.189, $p=0.43$

ALS severely affected (blue), Y = 0.533 − 0.02*X, R=-0.706, $p=0.015$

Controls (red), Y = 0.79 − 0.157*X, R=-0.810, $p<4 \times 10^{-5}$

B. Unit threshold vs rheobase in single motor units of ALS subjects (moderately affected, n=20; severely affected, n=11) and controls (n=21 units). Threshold is linearly correlated to rheobase in both ALS and NC and subjects:

ALS moderately affected (green), Y = -0.173 + 1.009*X, R=0.989, $p=6.4 \times 10^{-14}$

ALS severely affected (blue), Y = 0.199 + 0.951*X, R=0.991, $p=8.3 \times 10^{-8}$

Controls (red), Y = 0.219 + 0.800*X, R=0.977, $p=1.5 \times 10^{-11}$

Figure 5: Excitability of single motor axons in ALS.

Solid lines represent the mean, and dashed lines the standard error of the mean. Data from the ALS severely affected, ALS moderately affected and control groups are shown in blue, green and red, respectively. **A.** Threshold electrotonus recordings were significantly different for both depolarizing and hyperpolarizing current pulses (±20% and ±40% of control threshold). **B.** Recovery cycle. The relative refractory period was significantly shorter and superexcitability significantly greater in ALS motor units. **C.** Current-threshold relationship. The resting and minimal current-threshold slopes were significantly reduced in ALS motor units. The slope of the current-threshold relationship (***C***) is shown in ***D***, and more clearly demonstrates the reduction in resting and minimum current threshold slopes with disease progression (shown as Resting IV slope and Min. IV slope, respectively).

Figure 6: Correlation of clinical variables with Abnormal Excitability Index (AEI) in ALS motor units.

Moderately and severely affected ALS units are shown as green and blue circles, respectively. **A.** The abnormal excitability index was positive for 27 of the 31 ALS single motor units (the remaining four units exhibited a "notch" during depolarizing threshold electrotonus; see Text). The most positive values of AEI represent the most 'fanned-out' or abnormal recordings and roughly correspond to recordings from the more affected muscles. The severely affected axons were significantly more

abnormal than the moderate group (AEI: severe, 2.5± 0.6; moderate, 1.1 ± 0.2; *p*=0.0069. **B.** Strength measured using the MRC scale was negatively correlated with AEI, with the more abnormal single motor units innervating weaker muscles (Spearman rank correlation of all ALS units: ρ=-0.51, *p*=0.0034). **C.** The ALSFRS-fine subscore was also negatively correlated with AEI, the more abnormal the motor unit AEI the more impaired were fine motor skills (Spearman rank correlation of all ALS units: ρ=-0.55, *p*=0.0019).

Figure 7: Mathematical model of the excitability of ALS single motor units.

The mean excitability recordings are shown as filled circles (ALS moderately affected, green; ALS severely affected, blue; controls, red). The blue, green and red lines represent the best-fit outputs of the mathematical model for ALS severe, ALS moderate and control single motor units. **A.** Threshold electrotonus. **B.** Recovery cycle. **C.** Current-threshold relationship. **D.** IV slope (derived from the slope of the current threshold relationship plot shown in *C*).

Figure 8: Correlation of measures of slow K$^+$ activity with single motor unit size. The moderately and severely affected ALS units are represented by green and blue circles, respectively. **A.** *RC(2-1)* and *S2 accommodation* are the most specific measures of slow K$^+$ function and are strongly correlated in the ALS single units (linear regression of all ALS units: Y = 0.677 × X + 2.845, R=0.89, *p*=9.8×10^{-8}). **B.** *S2 accommodation* was correlated with the single motor unit size (Y = 10.15 × log(X) + 24.03, R=0.49, *p*=0.0087). **C.** *RC(2-1)* was also correlated with single motor unit size (Y = 10.52 x log(X) + 22.3, R=0.57, p = 0.0033).

Linear regression and 95% confidence intervals represented as solid and dashed lines respectively.