Cortical Function in Asymptomatic Carriers and Patients With C9orf72 Amyotrophic Lateral Sclerosis

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IMPORTANCE The identification of the chromosome 9 open reading frame 72 (c9orf72) gene hexanucleotide repeat expansion represents a major advance in the understanding of amyotrophic lateral sclerosis (ALS) pathogenesis. The pathophysiological mechanism by which the c9orf72 gene expansion leads to neurodegeneration is not yet elucidated. Cortical hyperexcitability is potentially an important pathophysiological process in sporadic ALS and familial ALS (FALS).

OBJECTIVE To investigate whether cortical hyperexcitability forms the pathophysiological basis of c9orf72 FALS using the threshold-tracking transcranial magnetic stimulation technique.

DESIGN, SETTING, AND PARTICIPANTS Prospective case-control single-center study that took place at hospitals and outpatient clinics from January 1, 2013, to January 1, 2015. Clinical and functional assessments along with transcranial magnetic stimulation studies were taken on 15 patients with c9orf72 FALS and 11 asymptomatic expansion carriers of c9orf72 who were longitudinally followed up for 3 years. Results were compared with 73 patients with sporadic ALS and 74 healthy control participants.

MAIN OUTCOMES AND MEASURES Cortical excitability variables, including short-interval intracortical inhibition, were measured in patients with c9orf72 FALS and results were compared with asymptomatic c9orf72 carriers, patients with sporadic ALS, and healthy control participants.

RESULTS Mean (SD) short-interval intracortical inhibition was significantly reduced in patients with c9orf72 FALS (1.2% [1.8%]) and sporadic ALS (1.6% [1.2%]) compared with asymptomatic c9orf72 expansion carriers (10.2% [1.8%]; F = 16.1; P < .001) and healthy control participants (11.8% [1.0%]; F = 16.1; P < .001). The reduction of short-interval intracortical inhibition was accompanied by an increase in intracortical facilitation (P < .01) and motor-evoked potential amplitude (P < .05) as well as a reduction in the resting motor threshold (P < .05) and cortical silent period duration (P < .001).

CONCLUSIONS AND RELEVANCE This study establishes cortical hyperexcitability as an intrinsic feature of symptomatic c9orf72 expansion-related ALS but not asymptomatic expansion carriers.
Identification of increased hexanucleotide repeat expansion (GGGGCC) in the chromosome 9 open reading frame 72 (c9orf72) gene,1,2 which appears to underlie more than 40% of familial amyotrophic lateral sclerosis (FALS) and 8% of sporadic amyotrophic lateral sclerosis (SALS) cases,1,3 has radically altered the understanding of ALS pathogenesis, broadening the clinical heterogeneity of ALS. The c9orf72 hexanucleotide expansion underlies both ALS and frontotemporal dementia (FTD).1,2 Subtle cognitive abnormalities may be evident in as many as 50% of patients with ALS and FTD may develop in 15% of patients with ALS.4

The precise pathophysiological mechanisms by which the c9orf72 gene expansion mediates neurodegeneration in ALS has not been established,1,2 although a number of pathogenic mechanisms have been proposed, including haploinsufficiency,1,2,5 RNA-mediated toxicity,6,7 and dipeptide repeat protein toxicity related to non-ATG (RAN) translation of the expanded c9orf72 gene.8,9 Motor neuronal hyperexcitability was suggested as a potential pathophysiological mechanism in c9orf72 ALS, a process mediated by the inactivation of K+ channels.10 Importantly, a blockade of neuronal hyperexcitability by retigabine, a K+ channel activator, exerted neuroprotective benefits, identifying a putative therapeutic target in ALS.10

In patients with ALS, motor neuronal hyperexcitability may be assessed by transcranial magnetic stimulation (TMS) techniques.4 Previous TMS studies in patients with SALS and patients with FALS linked to mutations in the superoxide dismutase 1 gene have established cortical hyperexcitability as an early feature in ALS linked to the process of neurodegeneration while the level of cortical excitability was normal in asymptomatic superoxide dismutase 1 gene mutation carriers.12-15 These studies highlighted the pathogenic importance of cortical hyperexcitability in ALS and suggested that the onset of ALS was potentially triggered by 1 or more factors associated with genetic mutations. Consequently, the present study used threshold-tracking TMS techniques to assess cortical function in asymptomatic c9orf72 expansion carriers, with results compared with patients with c9orf72 FALS and patients with ALS to clarify the underlying pathophysiological mechanisms in this form of FALS.

Methods

Patients

Patients were recruited from the ALS genetic database and multidisciplinary ALS clinics. Studies were undertaken on 15 patients with FALS (7 women and 8 men; mean age, 60 years; range 41-78 years), with a confirmed c9orf72 hexanucleotide expansion defined as possible or probable/definite ALS according to the Awaji criteria.16 In addition, studies were also undertaken on 11 asymptomatic c9orf72 mutation carriers (10 women and 1 man; mean age, 49 years; range 26-78 years) who were followed up for 3 years. Seventy-three patients with SALS were also assessed (45 men and 28 women; mean age, 60 years; range, 28-86 years).

Patients with ALS were clinically reviewed on a regular basis throughout the multidisciplinary ALS clinics. All patients were clinically staged using the Amyotrophic Lateral Sclerosis Functional Rating Scale–Revised17 and categorized according to the site of disease onset as limb- or bulbar-onset ALS. In addition, the disease duration (months) at the time of testing was recorded and muscle strength was assessed using the Medical Research Council (MRC) rating scale, with the following muscle groups assessed bilaterally, yielding a total MRC score of 90: shoulder abduction, elbow flexion, elbow extension, wrist dorsiflexion, finger abduction, thumb abduction, hip flexion, knee extension, and ankle dorsiflexion.

The degree of upper motor neuron dysfunction was assessed by a specific upper motor neuron score.18 None of the patients with ALS were receiving medications, which could have potentially interfered with neurophysiological results. Patients provided written informed consent to the procedures. The study was approved by the Sydney West Area Health Service and Human Research ethics committees.

Neurophysiological Studies

Cortical excitability was assessed by using a threshold-tracking TMS technique according to a previously reported method.19 The motor-evoked potential (MEP) response was recorded in the abductor pollicis brevis muscle. The following parameters were recorded in all participants: short-interval intracortical inhibition (SICI, %) between the interstimulus intervals (ISIs) of 1 to 7 milliseconds, intracortical facilitation (ISI of 10-30 milliseconds), resting motor threshold (%), MEP amplitude (%), cortical silent period duration (milliseconds), and central motor conduction time (milliseconds).

In the same sitting, nerve conduction studies and needle electromyography were undertaken on all participants. The compound muscle action potential (CMAP) was recorded from the abductor pollicis brevis muscle and the CMAP onset latency and peak-peak amplitude were measured. Subsequently, the neurophysiological index (NI) was derived according to a previously reported formula.20 Recordings of the compound muscle action potential (CMAP) and MEP responses were amplified and filtered (3 Hz-3 kHz), using a Cardinal Health Viking Select, version 11.1.0, Niolet-Biomedical EA-2 amplifier (Viasys Healthcare Neurocare Group) and sampled at 10 kHz using a 16-bit data acquisition card (National Instruments PCI-MIO-16E-4). Data acquisition and stimulation delivery were controlled by QTRACS software. Temperature was monitored with a purpose-built thermometer at the stimulation site.

Statistical Analysis

Cortical excitability was compared with 74 healthy control participants (37 men and 37 women; mean age, 53.1 years; range, 23-83 years). Data were assessed for normality using the Shapiro-Wilk test. The t test and Wilcoxon-Signed rank test were used to assess differences between means. Analysis of variance with post hoc testing using a Bonferroni correction (parametric data) or Kruskal-Wallis test (nonparametric data) were used for multiple comparisons. A value of P < .05 was considered statistically significant. A potential caveat was that healthy control participants were significantly younger than patients with c9orf72 expansion-related and SALS. Pearson or...
Spearman correlations were used to assess the relationship between parameters. Results are expressed as the mean (SD) or median with the interquartile range (IQR).

**Results**

**Clinical Features**

The clinical phenotype and level of functional impairment was similar between the 15 patients clinically affected with FALS and SALS. Specifically, the mean (SD) Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised score in patients with c9orf72 FALS was 39.4 (10.1), indicating a moderate degree of impairment and was comparable with patients with SALS (41.2 [5.4]; P = .25). In addition, the mean (SD) MRC total score in patients with c9orf72 FALS was 82.6 (7.1), reaffirming a moderate degree of functional impairment and was comparable with the MRC total score in patients with SALS (80.7 [11.5]; P = .09). The median upper motor neuron score was similar in patients with c9orf72 FALS (median, 14; IQR, 10-14; SD, 6) and SALS (median, 12; IQR, 10-14; SD, 5; P = .32), signifying a presence of upper motor neuron signs in both cohorts.

At the time of TMS testing, the median disease duration in patients with FALS was 8 months (IQR, 5-12 months; SD, 12.6 months) and was comparable with patients with SALS (median, 10.5 months; IQR, 6-18 months; SD, 18.1 months). Of the patients with FALS, 40% exhibited bulbar-onset disease while 60% reported limb-onset disease, identical to that in patients with SALS. All patients were receiving riluzole at the time of assessment.

In contrast, physical examinations in all the asymptomatic c9orf72 hexanucleotide expansion carriers, including the presymptomatic carrier, were normal. Specifically, there were no upper motor neuron features, such as increased muscle tone, hyperreflexia, extensor plantar responses, positive Hoffman sign, or the presence of a jaw jerk in any patients at the time of testing.

**Neurophysiological Studies**

The peripheral disease burden was assessed prior to undertaking cortical excitability studies. The CMAP amplitude (patients with FALS, 6.3 mV [2.3 mV]; patients with SALS, 7.0 mV [4.0 mV]; control participants, 9.9 mV [4.3 mV]; F = 6.6; P < .001) and NI (patients with FALS, 1.5 [1.2]; patients with SALS, 1.2 [0.9]; control participants, 2.3 [0.9]; F = 10.3; P < .001) were significantly reduced in patients with FALS and patients with SALS when compared with control participants. In contrast, there was no significant difference in the CMAP amplitude and NI between asymptomatic c9orf72 expansion carriers and control participants (CMAP for asymptomatic carriers, 9.2 mV [3.3 mV]; CMAP for control participants, 9.9 mV [4.3 mV]; P = .33; NI for asymptomatic carriers, 1.8 [1.3]; NI for control participants, 2.3 [0.9]; P = .20).

**Cortical Excitability**

Short-interval intracortical inhibition, reflected by an increase in the conditioned stimulus intensity required to track a constant target MEP of 0.2 mV, was significantly reduced in patients with FALS and patients with SALS (Figure 1A). The mean (SD) SICI, between ISI 1 and 7 milliseconds, was significantly reduced in patients with FALS and ALS when compared with control participants (patients with FALS, 1.2% [7.0%]; patients with SALS, 1.6% [10.3%]; control participants, 11.8% [8.6%]; FI = 16.1, P < .001; Figure 1B) as was the SICI at ISI of 1 millisecond (patients with FALS, 0.3% [7.0%]; patients with SALS, 1.7% [6.8%]; control participants, 6.8% [10.3%]; FI = 4.9; P < .005; Figure 2A) and SICI at ISI of 3 milliseconds (patients with FALS, 4.1% [9.3%]; patients with SALS, 3.5% [13.7%]; control participants, 17.2% [12.9%]; FI = 18.1; P < .001; Figure 2B). In contrast, there was no significant difference in the mean (SD) SICI (asymptomatic carriers, 10.2% [6.0%]; control participants, 11.8% [8.6%]; PI = .20; Figure 1B), SICI at ISI of 1 millisecond (asymptomatic carriers, 5.6% [6.6%]; control participants, 6.8% [10.3%]; P = .20; Figure 2A), and SICI at ISI of 3 milliseconds (asymptomatic carriers, 15.5% [9.6%]; control participants, 17.2% [12.9%]; FI = 18.1; P < .001; Figure 2B).
control participants, 17.2% [12.9%]; P = .10; Figure 2B) between asymptomatic c9orf72 carriers and control participants.

Following SICI, a period of intracortical facilitation developed between an ISI of 10 and 30 milliseconds. Mean (SD) intracortical facilitation was increased in patients with FALS and patients with SALS (patients with FALS, −3.2% [4.3%]; patients with SALS, −4.1% [6.0%]; F = 3.3; P < .05; Figure 3A) when compared with control participants (−1.4% [6.9%]). In contrast, mean (SD) intracortical facilitation was comparable between the asymptomatic expansion carriers (−1.2% [2.0%]; P = .40) and control participants.

Single-pulse TMS disclosed a significant increase in the MEP amplitude expressed as a percentage of CMAP response in both patients with FALS and patients with SALS when compared with control participants (patients with FALS, 45.2% [25.5%]; patients with SALS, 32.2% [23.1%]; control participants, 23.4% [13.8%]; F = 3.5; P < .01; Figure 3B). In contrast, the MEP amplitude in asymptomatic expansion carriers was similar to control participants (asymptomatic expansion carriers, 24.4% [12.9%]; control participants, 23.4% [13.8%]; P = .42). In addition, the cortical silent period duration was significantly reduced in patients with FALS and patients with SALS (patients with FALS, 186.1 milliseconds [40.7 milliseconds]; patients with SALS, 173.8 milliseconds [41.9 milliseconds]; control participants, 214.1 milliseconds [33.6 milliseconds]; F = 10.8; P < .001; Figure 3C) but not in the asymptomatic expansion carriers (211.4 milliseconds [44.1 milliseconds]; P = .40).

Of further relevance, the resting motor threshold was significantly reduced in patients with c9orf72 FALS (52.2% [8.1%]) when compared with SALS, asymptomatic carriers, and control cohorts (patients with SALS, 57.2% [11.1%]; asymptomatic carriers, 58.5% [16.6%]; control participants, 60.3% [12.0%]; F = 3.4; P < .05). In addition, the central motor conduction time was significantly increased in patients with FALS and SALS (patients with FALS, 5.9 milliseconds [1.9 milliseconds]; patients with SALS, 6.6 milliseconds [1.7 milliseconds]; control participants, 5.5 milliseconds [2.6 milliseconds]; F = 4.4; P < .01, Figure 3D). In contrast, the central motor conduction time was not significantly increased in asymptomatic carriers (asymptomatic carriers, 5.2 milliseconds [2.0 milliseconds]; control participants, 5.5 milliseconds [2.6 milliseconds]; P = .30).

Correlation Studies
By combining clinical, peripheral, neurophysiological, and cortical excitability findings in the c9orf72 FALS cohort, the mean SICI (ISI, 1-7 milliseconds) significantly correlated with the CMAP amplitude (R = −0.55; P < .05), MRC upper limb score (ρ = −0.80; P < .01), and the MRC abductor pollicis brevis score (ρ = −0.46; P < .05). In addition, intracortical facilitation significantly correlated with the CMAP amplitude (R = −0.67; P < .05) as did the cortical silent period duration (R = −0.59; P < .05) and resting motor threshold (R = 0.53; P < .05). Taken together, these findings suggest the features of cortical hyperexcitability were most prominent when muscle strength and motor amplitudes were relatively preserved.

Discussion
The findings in the present study confirmed cortical hyperexcitability as an intrinsic feature of c9orf72 expansion-related ALS as well as SALS. Cortical hyperexcitability was associated by a marked reduction of short-interval intracortical inhibition and cortical silent period duration combined with an increase in MEP amplitude and intracortical facilitation. In contrast, cortical excitability was normal in the asymptomatic c9orf72 expansion carriers and was significantly different when compared with FALS and SALS cohorts. The potential mechanisms underlying these findings and their pathophysiological implications for ALS are further discussed.

Cortical Hyperexcitability and ALS Pathogenesis
Pathophysiological mechanisms underlying the development of motor neuron degeneration in ALS appear to be mul-
tifactorial, with a complex interaction between genetic factors and the dysfunction of vital molecular pathways.

The identification of the dominantly inherited c9orf72 gene as an important genetic etiology of ALS1,2 has altered the understanding of ALS pathogenesis, building on the concept of ALS as a multisystem neurodegenerative disorder22 and emphasizing the importance of cortical mechanisms in ALS pathophysiology. This was further underscored by neuropathological studies demonstrating cortical intraneuronal inclusions evident in ALS associated with c9orf72 and frontotemporal dementia.23

Cortical hyperexcitability was a feature of c9orf72 FALS comparable with findings in the SALS cohort and evidenced by a marked reduction of SICI. Short-interval intracortical inhibition is mediated by the activation of cortical inhibitory circuits that act via γ-aminobutyric acid type A receptors as well as glutaminergic neurotransmission.11,24,25 Consequently, the reduction of SICI could have been mediated by the degeneration and dysfunction of inhibitory cortical interneurons26,27 as well as glutamate-mediated excitotoxicity. A significant reduction in the cortical silent period duration and resting motor thresholds along with an increase in the MEP amplitude further supported the presence of cortical hyperexcitability in patients with c9orf72 ALS.

The findings of cortical hyperexcitability in the present c9orf72 FALS cohort were similar to previous studies in patients with FALS attributed to different genetic mutations.13,28 As such, cortical hyperexcitability appears to represent a uniform pathophysiological process in ALS irrespective of underlying genetic status. Mathematical modeling has inferred a 6-step process in ALS,29 with a prolonged prodromal period potentially extending to the perinatal period.30 Cortical hyperexcitability could represent one of the final steps in ALS pathogenesis, perhaps developing just prior to or at the onset of neuronal degeneration, a notion supported by findings of significant correlations between features of cortical hyperexcitability, motor amplitude, and muscle strength. Animal studies support this assumption, identifying neuronal hyperexcitability at a preclinical stage upstream of the spinal motor neuron.31,32

Further supporting a multistep process in ALS is the finding of normal cortical excitability in the asymptomatic c9orf72 expansion carriers similar to the findings in asymptomatic superoxide dismutase-1 gene mutation carriers.13

Figure 3. Cortical Hyperexcitability as a Feature of C9orf72 Familial Amyotrophic Lateral Sclerosis (FALS)

A, Intracortical facilitation was significantly increased in patients with FALS and patients with sporadic ALS (SALS). B, Motor-evoked potential was significantly increased in patients with FALS and SALS when compared with asymptomatic c9orf72 expansion carriers and control participants. C, The cortical silent period duration was significantly reduced in patients with FALS and patients with SALS when compared with asymptomatic c9orf72 expansion carriers and control participants. D, Central motor conduction time was significantly increased in patients with FALS and patients with SALS. All P values were corrected for multiple comparisons using post hoc Bonferroni testing.

a P < .05.

b P < .01.

b P < .001.
Consequently, the development of neuronal hyperexcitability in FALS appears to be dependent on factors other than the inheritance of genetic mutations. Identifying and modulating these triggering factors could be therapeutically significant for ALS. In addition, the findings of normal cortical excitability in the asymptomatic FALS cohorts would argue against therapeutic benefits of prophylactic riluzole.

Conclusions
It could be argued that cortical hyperexcitability was an adaptive process in response to peripheral neurodegeneration and could serve as a neuroprotective strategy. Underscoring this notion are recent animal studies suggesting that an increase in neuronal excitability may be neuroprotective.11 While this cannot be discounted in patients with ALS, it seems unlikely, given that riluzole, an antiglutaminergic agent that prolongs survival,33,34 reduces cortical hyperexcitability.25 In addition, the reduction of neuronal hyperexcitability by pharmacological agents, such as retigabine, appears to be neuroprotective,10 while normalizing astrocyte function by knocking down the expression of mutated genes and, therefore, normalizing glutamate homeostasis, appears to be neuroprotective.29 In addition, features of cortical hyperexcitability were not evident in non-ALS neuromuscular cohorts despite a comparable degree of peripheral neurodegeneration,14,15,36 further arguing against the notion that cortical hyperexcitability represents a simple compensatory mechanism. Rather, cortical hyperexcitability may serve as a final common pathway in ALS, mediating neuronal degeneration via a transsynaptic glutamate process. The identification and modulation of factors that trigger cortical hyperexcitability may prove therapeutically useful.


