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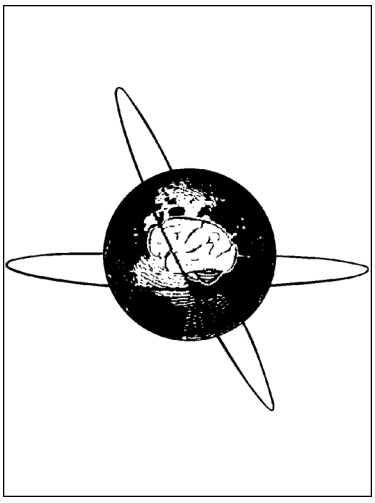

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Interrogating Interneurone Function using Threshold Tracking of the H Reflex in Healthy Subjects and Patients with Motor Neurone Disease

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Keywords: presynaptic inhibition, H reflex, motor neurone disease, amyotrophic lateral sclerosis, interneuronopathy, threshold tracking.

HIGHLIGHTS

1. Threshold-tracking H reflexes addresses some of the problems with traditional methods for studying motoneurone excitability.
2. Presynaptic inhibition of the H reflex was lower in motor neurone disease, possibly due to an interneuronopathy.
3. Hyperreflexia could reflect a spinal pre-motoneuronal abnormality, and may not be definitive evidence of corticospinal involvement.

ABBREVIATIONS:

- ALSFRS-R: ALS Functional Rating Scale
- D1: second phase of suppression of the soleus H reflex produced by group Ia afferents from the pretibial flexor muscles, maximal ~20 ms after the conditioning stimulus, used as a measure of presynaptic inhibition,
- D2: third phase of suppression of the soleus H reflex produced by group Ia afferents from the pretibial flexor muscles, maximal at ~200 ms and separated from D1 by an phase of excitation but also largely due to presynaptic inhibition.
- H_{max}: the largest H reflex response
- M_{max}: the maximal motor response, i.e., the maximal M wave due to direct stimulation of motor axons
- MRC: UK Medical Research Council
- PLS: primary lateral sclerosis
- xMT: times motor threshold (i.e., the threshold for a liminal M wave)

ABSTRACT

***Objective*:** The excitability of the lower motoneurone pool is traditionally tested using the H reflex and a constant-stimulus paradigm, which measures changes in the amplitude of the reflex response. This technique has limitations because reflex responses of different size must involve the recruitment or inhibition of different motoneurones. The threshold-tracking technique ensures that the changes in excitability occur for an identical population of motoneurones. We aimed to assess this technique and then apply it in patients with motor neurone disease (MND).

Methods: The threshold-tracking approach was assessed in 17 healthy subjects and 11 patients with MND. The soleus H reflex was conditioned by deep peroneal nerve stimulation producing reciprocal Ia and so-called D1 and D2 inhibitions, which are believed to reflect presynaptic inhibition of soleus Ia afferents.

***Results*:** Threshold tracking was quicker than the constant-stimulus technique and reliable, properties that may be advantageous for clinical studies. D1 inhibition was significantly reduced in patients with MND.

***Conclusions*:** Threshold tracking is useful and may be preferable under some conditions for studying the excitability of the motoneurone pool. The decreased D1 inhibition in the patients suggests that presynaptic inhibition may be reduced in MND.

Significance: Reduced presynaptic inhibition could be evidence of an interneuronopathy in MND. It is possible that the hyperreflexia is a spinal pre-motoneuronal disorder, and not definitive evidence of corticospinal involvement in MND.

INTRODUCTION

In motor control studies on human subjects, it is usual to assess changes in the excitability of a motoneurone pool by measuring the changes in size of the compound muscle action potential produced by the H reflex. Excitation of the motoneurone pool causes more motoneurones to discharge in response to a constant test stimulus, thus increasing the size of the reflex response, while inhibition decreases the size of the reflex response. There are a number of reservations limiting the interpretation of changes in the compound H reflex, and these are collectively known as pool problems.

First, there is non-linearity in the input-output response, even when it is expressed as a percentage of the maximal compound muscle action potential, M_{max} (Crone et al. 1990). Consequently, when a manoeuvre such as a voluntary contraction increases the size of the test reflex, it is unlikely that a conditioning volley will produce the same percentage change in the test reflex.

Secondly, the reflex effects of some inputs are not homogeneous across the pool, and can have different effects on low-threshold and high-threshold motoneurones. For example, in the decerebrate cat sural volleys can inhibit low-threshold motoneurones while exciting high-threshold motoneurones, thus reversing the normal order of recruitment (Kanda et al. 1977). Similar findings have been documented in the human hand (Garnett and Stephens, 1981). As a result, when a test H reflex is conditioned by a cutaneous volley, the size of the test H reflex may determine whether you see inhibition or facilitation.

Thirdly, there can be changes in the input/output gain of the motoneurone pool, such that an afferent input produces a different output even when the same motoneurones are active. An example of this is the ability of different manoeuvres to activate persistent inward currents that amplify and outlast the original stimulus (ElBasiouny et al., 2010). Some reports indicate that persistent inward currents are upregulated in animal models of amyotrophic lateral sclerosis (Meehan et al., 2010; Quinlan et al., 2011), though there is evidence against this from both a mouse model of inherited

amyotrophic lateral sclerosis (Delestrée et al., 2014) and patients with the disease (Marchand-Pauvert et al., 2019). Either way, this is relevant to our choice of the patient group used to validate threshold tracking of the H reflex for clinical studies.

One approach to the first two pool problems would be to clamp the motoneurone discharge so that its size could not vary. Studies on single motor units have been used to complement studies of the compound H reflex (Pierrot-Deseilligny and Burke, 2012), the rationale being that, if the same behaviour can be reproduced in a single motoneurone, non-linearity of the pool's response can be excluded as the cause of the findings with the compound H reflex. This approach is possible only with low-threshold motoneurones, and their behaviour does not always reflect that of the whole pool (*see* above). An alternative method would be to adapt the technique of threshold tracking (Bostock et al., 1998; Burke et al., 2001) to the H reflex, varying the stimulus strength so that the size of the H reflex remains constant despite changes in motoneurone excitability. The same population of motoneurones can then be studied, despite shifts in the excitability of the pool. This technique has been used to study the strength-duration properties of Ia afferents responsible for the H reflex (Lin et al., 2002), the effects of paired-pulse stimulation on the H reflex (Chan et al., 2002), and post-activation depression of the H reflex (McNulty et al., 2008). Threshold tracking has not been used previously to document the changes in motoneurone excitability produced by a conditioning stimulus delivered to a heteronymous nerve, but this is how interneuronal function is studied in human subjects (Pierrot-Deseilligny and Burke, 2012).

An aim of the present study was therefore to examine the feasibility and utility of this approach for documenting interneuronal function by studying the inhibitory effects of volleys in group Ia afferents in the deep peroneal nerve on the excitability of soleus motoneurones. The threshold tracking results are compared with those of the constant-stimulus method in which the changes in size of the H reflex are used as a measure of motoneurone excitability. We then used threshold tracking to determine whether these inhibitory reflex effects are altered in motor neurone disease (MND). We report

evidence for decreased presynaptic inhibition in the patients with MND, possibly due to an interneuronopathy.

METHODS

Subjects. Seventeen healthy subjects and 11 patients with MND participated in the study. All were studied at the Brain & Mind Centre or the Medical Foundation Building of the University of Sydney. The ages were for controls: mean 42.5 years (SD 11.8) and for the patients: mean 55 years (SD 13.5). All participants gave informed written consent to the experimental procedures, which were approved by the Human Ethics Committee of the University of Sydney (2014/056) and conformed to the requirements of the *Declaration of Helsinki*. Of the 11 patients diagnosed with MND (Table 1), 10 had or have since developed into probable or definite amyotrophic lateral sclerosis (ALS) according to the revised El-Escorial criteria (Brooks et al., 2000; Kiernan et al., 2011) and one had primary lateral sclerosis (PLS). The first symptoms appeared 6-62 months before the study for the 10 patients with ALS and 120 months earlier in the patient with PLS. Two of the 11 patients in Table 1 (#3, initially classified as possible ALS, and #5, probable ALS) died of ALS 45 and 27 months after testing, respectively. Genetic testing is undertaken in all MND patients that attend this clinic, given the frequency of genetic abnormalities evident in apparently sporadic MND patients (Blair et al., 2010), particularly the presence of *C9orf72* abnormalities (Williams et al, 2013). None of the patients harboured known mutations associated with MND/ALS.

Forty-three experiments were performed in the 28 subjects (healthy controls and patients). In the healthy subjects, the effects of peroneal nerve conditioning on the H reflex of soleus was measured using both the threshold tracking and constant-stimulus paradigms. These studies were repeated in four healthy subjects, on both sides in 3 subjects and on the same side in one subject. Additional experiments were undertaken to measure changes in the H reflex recruitment curve produced by the

conditioning stimuli in 5 healthy subjects, and the effects of three conditioning stimuli at 3-ms intervals were assessed in 5 healthy subjects and one patient. The flowchart in Figure 1 illustrates the experiments performed on the control subjects. Single experiments were then performed on the 11 patients using threshold tracking to measure the changes in excitability of soleus motoneurones produced by conditioning stimuli to the deep peroneal nerve. Control subjects and patients with small H reflexes had to be excluded from the study because of issues with threshold tracking (5 subjects, 6 experiments; *see* below).

Testing presynaptic inhibition in human subjects. As discussed elsewhere (Pierrot-Deseilligny and Burke, 2012), presynaptic inhibition of group Ia afferents projecting to soleus involves a presynaptic inhibitory interneurone that makes an axo-axonal synapse with the terminals of the Ia afferent and uses gamma aminobutyric acid as the transmitter. This interneurone is excited or inhibited by peripheral afferents, dependent on their site of origin, and by descending pathways from cerebral sources. Activation of this interneurone down-regulates the group Ia excitation of the motoneurone without altering its excitability. There are two reliable techniques for studying presynaptic inhibition in human subjects (Pierrot-Deseilligny and Burke, 2012). First, the interneurone can be activated by group Ia volleys from the antagonist (Hultborn et al., 1987). The greater the inhibition, the greater the presynaptic inhibition. This is the method chosen for the present study, though here we used electrical stimulation of Ia afferents in the deep peroneal nerve. Secondly, the soleus H reflex can be conditioned by group Ia afferents in the femoral nerve, thus producing heteronymous monosynaptic excitation of soleus motoneurones. The extent heteronymous monosynaptic excitation is inversely related to the background presynaptic inhibition of afferents projecting to soleus – the smaller the excitation the greater the presynaptic inhibition (Hultborn et al., 1987).

Experimental procedures. Subjects were seated in a comfortable chair with the hip and knee flexed by 30 degrees. The H reflex of soleus was elicited by stimulation of the tibial nerve in the popliteal fossa using two Digitimer DS5 Isolated Bipolar Current Stimulators (Digitimer, Welwyn Garden

City, UK) connected in parallel. The cathode was over the popliteal fossa and the anode was on the anterior aspect of the leg below the patella. Stimuli were delivered once every 3 s in blocks with intermittent rest periods. To minimise homosynaptic depression the responses to the first four stimuli after a rest period were discarded, and data were then collected as described below. The method for measuring changes in the H reflex (*i.e.*, using threshold tracking or the constant-stimulus method) was alternated, and measurements were made in an orderly sequence of incremental conditioning-test intervals, with 3-4 successive intervals in each recording block. For the constant-stimulus method, 20 measurements were made of the conditioned H reflex at each conditioning-test interval. In each sequence, stimuli for the H reflex were unconditioned (control) or conditioned, delivered in the following pseudo-random sequence, such that 5 unconditioned (U) and 5 conditioned (C) stimuli were delivered every 30 s:

U,C,U,U,C,C,U,C,C,U,C,U,C,C,U,U,U,U,C,U,C,C,U,U,C,C,U

The electromyographic activity (EMG) of soleus was recorded by surface electrodes 4 cm apart over soleus, distal to the two heads of the gastrocnemius muscles. EMG was amplified ×200, filtered 3 Hz – 3 kHz, and fed through a Hum Bug 50/60 Hz Noise Eliminator (Quest Scientific Instrument Co., North Vancouver, Canada). Stimulus delivery and digitisation of the amplified EMG were controlled by QtracS (© Prof Hugh Bostock, Institute of Neurology, UCL, UK), using threshold tracking software (Kiernan et al., 2020) and a multi-purpose data acquisition system (National Instruments, USB-6251). A recruitment curve was measured in each experiment to document the sizes of the maximal H reflex (H_{max}) and of the maximal direct motor response (M_{max}).

For the ***constant-stimulus paradigm***, the intensity of stimulation was set to evoke a test H reflex that was 20% of the M_{max}. Changes in the amplitude of this response were taken as an indication of the change in motoneurone excitability induced by the peroneal conditioning stimuli.

Threshold tracking paradigm. The stimulus to the tibial nerve was altered in 0.125% steps by computer until the reflex amplitude oscillated about the target size, usually 20% M_{max}, ensuring that, as far as was possible, the reflex response did not exceed 50-60% H_{max}. In Figure 2, the reflex potential in the thick trace was larger than the target, and the next stimulus was therefore decreased; the reflex potential in the thin trace was smaller, and the next stimulus was therefore increased. For the 3.5 ms conditioning-test interval, the direct motor response to the conditioning stimulus (1.4 times motor threshold) appeared in the pretibial flexor muscles and could be recorded over soleus. For the 21-ms interval, this far-field potential ended before the test H reflex and was smaller because the conditioning stimulus was 1.2 times motor threshold.

Measurements of the conditioned H reflex were made when the recorded H reflex was within 10% of the target size (*i.e.*, between 18% and 22% of M_{max} when the target size was 20% M_{max}), or when no more than 2 responses were on the same side of the target. Eight measurements were then made at each conditioning-test interval.

Conditioning stimuli were of 1-ms duration, delivered using a Digitimer DS5 Stimulator to the deep peroneal nerve at the fibular head, taking care to ensure that the direct motor response occurred in the pretibial flexors and not the peroneal muscles. The conditioning stimuli produced 3 phases of inhibition of the soleus H reflex, as described by others (Mizuno et al., 1971; El-Tohamy and Sedgwick, 1983; Iles and Roberts, 1987; Iles, 1996; Faist et al., 1996; Capaday et al., 1995; Aymard et al., 2000). At conditioning-test intervals up to 6 ms, conditioning stimuli were 1.4 times motor threshold to document short-latency reciprocal Ia inhibition. From 7 ms, conditioning stimuli were 1.2 times motor threshold to document the longer-latency D1 and D2 inhibitions (which are presumed to result from presynaptic inhibition of Ia afferents projecting to soleus; *see* Mizuno et al., 1971; El-Tohamy and Sedgwick, 1983; Iles and Roberts, 1987; Iles, 1996; Faist et al., 1996; Capaday et al., 1995; Aymard et al., 2000). Data were collected at intervals focused on the expected inhibitory effect (at 2-6 ms for reciprocal Ia

inhibition; at 11-50 ms for D1 and then at 10 ms intervals up to 100 ms and thereafter at 100 ms intervals to 500 ms for D2).

In the majority of experiments, conditioning stimuli were single shocks (as used by Iles and Roberts, 1987; Iles, 1996; Morita et al., 2001). Because others have studied D1 and D2using trains of stimuli (*e.g.*, 3 at 200 or 300 Hz; *see* El-Tohamy and Sedgwick 1983; Aymard et al., 2000), experiments were performed in 5 control subjects and 1 patient using a conditioning train of 3 identical stimuli at 3-ms intervals. The inhibitory responses were not any clearer in these experiments, and they will not be detailed further.

Additional experiments. The motoneurones involved in the inhibitory response were explored in five subjects. The full H reflex recruitment curve was measured using the constant-stimulus paradigm, under control conditions without preceding conditioning, and when the test stimuli were preceded by conditioning stimuli at 3.5 ms (for reciprocal Ia inhibition), 21 ms (for D1) and 200 ms (for D2).

Analysis. To compare the 2 techniques for measuring the excitability of the motoneurone pool and to compare threshold tracking data for control subjects and patients, recovery curves were constructed from 1 ms to 500 ms. For the 4 control subjects on whom more than one experiment was performed, the data were averaged, given that there are minor if any side-to-side differences for the tested properties (Pierrot-Deseilligny and Burke, 2012). To examine which motoneurones were involved in the inhibitory phases, the unconditioned stimulus-response curves were subtracted from the conditioned stimulus-response curves, and a cumulative sum of the differences was generated, threshold being that for a liminal direct motor response in soleus. Because the precise latency at which each inhibitory effect was maximal was slightly different in different subjects, the areas of the inhibitory phases were calculated over 3-4 ms for reciprocal Ia inhibition, 11-21 ms for D1 and 100-300 ms for D2. To demonstrate an effect of the interpolated facilitation between D1 and D2 on the size D2, the amplitude of the facilitation was regressed against the size of D2, measured for each individual where the responses were maximal. Data are given in the text and figures as mean (standard deviation). The

significance of differences in normally distributed measures (recording times; mean data) were calculated using Student's t test.

RESULTS

Comparison of two methods for measuring the inhibitory phases in healthy subjects. The 2 methods of documenting the changes in motoneurone excitability provided essentially similar results (Fig. 3). There was a short-latency inhibitory phase maximal at 3-4 ms (referred to here as "reciprocal Ia inhibition"), a second phase of inhibition (D1) maximal at ~20 ms, followed by a phase of increased excitability at ~40 ms, and then a deeper phase of inhibition maximal at 100—200 ms (D2). The time taken for threshold tracking was significantly shorter than for the constant-stimulus method for each inhibitory phase (Fig. 4): reciprocal Ia inhibition: 9.7 min (SD 1.3) *vs* 16.2 min (SD 0.3); for D1: 11.4 min (SD 2.0) *vs* 18.6 min (SD 0.3); for D2 13.5 min (SD 2.4) *vs* 20.5 min (SD 0.4), $P < 5 \times 10^{-24}$, 2×10^{-19} and $6 \times 10^{-17,}$ respectively (two-tailed Student's t test). Threshold tracking took a longer time than the constant stimulus method for only 1 measurement in 1 subject (D2, red arrow in Fig. 4), and this was because profound inhibition caused such an increase in stimulus intensity that the H reflex fell on the falling phase of the recruitment curve. When the reflex response was on or near the falling phase of the stimulus-response curve, a small change in the reflex potential provoked a further increase in stimulus intensity and a further decrease in the reflex potential. The larger SDs for threshold tracking mirror the spread of data in Figure 4, and reflect the need for the stimulus to track up to the necessary level for each inhibitory phase. Despite the shorter recording time, the recovery cycle was smoother with threshold tracking and the between-subject variability of the averaged conditioned and control reflex responses were similar.

Which motoneurones are inhibited? To determine whether motoneurones are equally susceptible to the inhibitory mechanisms, the full stimulus-response curve was measured after conditioning stimuli delivered 3.5, 21 and 200 ms before the test stimulus (Fig. 5; unconditioned control:

black; reciprocal Ia: red; D1: blue; D2: green). These studies were performed using the constant-stimulus technique, measuring changes in the amplitude of the H reflex. The changes in the H reflex recruitment curves were small (Fig. 5) and were largely confined to its rising phase (left-hand panels). The inhibitory effects of the conditioning stimuli are clearer in the corresponding cumulative sum plots in the right-hand panels of Figure 5. These plots were derived by subtracting the unconditioned test responses from the appropriate conditioned responses and then summing the differences. For each subject, the 3 inhibitory phases began at the same % of M_{max}. The upper 2 subjects had quite large unconditioned H reflexes (H_{max} 80-90% of M_{max}), and in them the H reflex threshold was 0.6 times motor threshold. This corresponds to the threshold reported in other studies for detectable group Ia effects (Pierrot-Deseilligny and Burke, 2012). For the lower 2 subjects, H_{max} was a smaller percentage of M_{max}, and a greater afferent input was therefore required to produce reflex responses.

From these data, we conclude that the same motoneurones contribute to the 3 inhibitory phases - *i.e.*, that low-threshold motoneurones are susceptible to the processes underlying each of the inhibitory phases. The cumulative sums reached a plateau on the descending phase of the H reflex recruitment curve as the antidromic volley decreased the size of the reflex potential. It cannot be concluded that high-threshold motoneurones are less affected by the inhibition because higher-threshold motoneurones have larger axons that are likely to be preferentially activated by electrical stimuli. Collision between the antidromic motor volley and the reflex discharge would then eliminate any contribution of large motoneurones to the reflex potential.

Having validated the threshold tracking approach against the constant-stimulus paradigm, the utility of threshold tracking in a conditioning-test paradigm was explored in patients with MND.

Pattern seen in the patient group. A similar pattern was seen in the 11 patients with MND. However, in the averaged recovery curves of Figure 6, each inhibitory phase appeared smaller in the patients (open symbols) than in the control subjects (closed symbols). Figure 7 shows each inhibitory phase as a percentage of the threshold for the unconditioned H reflex. While each phase is smaller in

Fig. 6, only the reduction of the D1 phase was significant: reciprocal Ia inhibition -0.82 ± 2.5% *vs* -0.03 ± 1.8, *P* = 0.393, unpaired two-tailed t test; D1 -1.62 ± 1.45 *vs* -0.034 ± 1.10; *P* = 0.006; D2 -5.24 ± 6.33 *vs* -2.48 ± 2.56; *P* = 0.197; Fig. 7). When the data point for the outlier control subject in Figure 7 was deleted, the change in D1 was still significant (*P* = 0.009).

Effects of age on the inhibitory phases. Threshold tracking of the H reflex proved difficult in older subjects, and the 2 groups were not age-matched, the patients being significantly older than the control subjects (right panel, Fig. 7; *P*=0.016; unpaired 2-tailed t test). The H:M ratio decreases in healthy older subjects and the H reflex changes less in postural tasks than in young subjects (Tsuruike et al., 2003; Baudry and Duchateau, 2012; Baudry et al., 2015). In the present study, small reflexes <10% M_{max} proved unsuitable for threshold tracking. It is possible that the age difference between the 2 groups was, in part, because the upper motor neurone involvement in the patients produced a larger H reflex that was more suitable for threshold tracking.

To control for slight variability in the latency of the maximal inhibition in different subjects, the mean inhibition was calculated for each of the inhibitory phases (reciprocal Ia inhibition: 3-4ms; D1: 11-21 ms; D2: 100-200 ms) and was regressed against age. There were no significant relationships with age for either the controls or the patients (*e.g.*, for D1, the only inhibitory phase that was significantly different between the patients and the controls, *P*=0.552 and 0.519, respectively). We conclude that the decrease in D1 cannot be attributed to the greater age of the patient cohort.

Intervening excitation and its effect on D2. The peak of excitation between D1 and D2 varied in amplitude and latency for different subjects, and its amplitude was therefore measured for each subject at the latencies at which D1 and the excitatory peak were greatest. There was a significant correlation between the excitation and the extent of D2 in the patients (R^2 = 0.758; *P* < 0.001), but not in the control subjects (R^2 = 0.176; *P* < 0.0936). We conclude that the D2 inhibition is likely to be overestimated in patients, due to the decaying excitation.

Correlation with clinical features in the patient group. Table 1 provides data on clinical variables. There were no correlations between the sizes of the inhibitory phases and disability or other clinical features: Revised ALS Functional Rating Scale (ALSFRS-R, Cedarbaum et al., 1999), strength (Medical Research Council [MRC] Sum Score (*see* Kleyweg et al., 1991); MRC score for tibialis anterior, upper motor neurone score (Iwata et al., 2008)), or demographic variables (site of onset, time from onset of symptoms and gender). The absence of a relationship with upper motor neurone signs is not surprising (Swash, 2012; Huynh et al., 2016; Swash et al., 2020), given that lower motor neurone degeneration complicated the clinical picture in 10 of the 11 patients and dominated the disability in one patient.

DISCUSSION

The present study has assessed threshold tracking of the H reflex as a technique for documenting the excitability of the motoneurone pool in a conditioning-test paradigm. This method was compared in healthy subjects with the conventional constant-stimulus technique, where changes in the size of the reflex response occur when fewer or more motoneurones are recruited by the same stimulus. We have documented the advantages and limitations of the threshold-tracking technique, and these have been explored further in studies in patients with MND. We argue below that our findings in the patient group are consistent with a decrease in presynaptic inhibition in MND. We acknowledge a few reservations. MND is a heterogenous disorder, the sample sizes in this study were relatively small, and there was variability in the test outcomes in different subjects and patients. Patients were on riluzole, and some may have been taking self-prescribed agents.

Advantages and limitations of threshold tracking. Threshold tracking is more rapid (~60% of the time for the constant-stimulus method) and the results seem no more variable, properties that are shared with threshold tracking of the motor evoked potential produced by transcranial stimulation of

the motor cortex (Samusyte et al., 2018; Matamala et al., 2018b). In studies on patients, the duration of a test can be a limiting factor, and in this respect threshold tracking has a clear advantage. If subjects have a high H:M ratio (as did 2 subjects in Fig. 5), it would be possible to track the behaviour of reflex potentials of different size – *i.e.*, motoneurones of different threshold. However, only subjects with relatively large reflex responses can be studied, unless reflex size is stable. This precludes many older subjects, whether healthy or not. In addition, threshold tracking requires special software, equipment and expertise, not generally available even for motor control physiologists.

If the prime aim is to study the excitability of the motoneurone pool, threshold tracking addresses the major limitation of the constant-stimulus H reflex technique, namely that different motoneurones are being studied when there are changes in the size of the test reflex. The only other technique to address this issue involves studying the behaviour of single motor units, but these are of necessity the lowest-threshold motor units in the pool, even when selective recording techniques are used. As mentioned in the Introduction, higher-threshold units do not always respond similarly, *e.g.*, in response to cutaneous inputs (Garnett and Stephens, 1981).

The mechanisms of the inhibitory phases. The data in Figure 5 provide evidence that the three inhibitory phases involve similar motoneurones. Given the reflex pattern, the responsible afferents in the conditioning volley are presumably group I afferents from the pretibial flexors, specifically group Ia afferents. We cannot exclude a contribution from other mechanisms because the conditioning stimulus intensities were ≥ 1.2 times motor threshold. The central delay of disynaptic reciprocal inhibition is ~1 ms, but the conditioning stimulus was distal to the test stimulus in our experiments, by some 8 cm, accounting for an additional 1-2 ms. Theoretically, the conditioning stimulus intensity of 1.4 times motor threshold could have resulted in contamination of reciprocal inhibition by recurrent inhibition due to activation of Renshaw cells. However, in the cat, there is no recurrent inhibition between pure antagonists, such as tibialis anterior and soleus (Wilson et al., 1960; Hultborn et al., 1971). This also seems to be the case in humans in whom recurrent inhibition is generally linked quantitatively to

heteronymous Ia excitation, which does not occur between tibialis anterior and soleus (Pierrot-Deseilligny and Burke, 2012).

The D2 inhibition was preceded by a peak of facilitation which has been attributed by others to cutaneous pathways (*see* Pierrot-Deseilligny and Burke, 2012), and which overlaps the long-lasting presynaptic inhibition, and thereby prevents its true extent being measured. In the patients, the size of the facilitation was correlated with the extent of D2 but in the control subjects there was only a non-significant trend for this. A further issue with D2 as a measure of presynaptic inhibition is its duration – up to 500 ms for D2 (*see* Figs 3 and 6) but <300 ms for presynaptic inhibition.

D1 was maximal at ~20 ms, and it is difficult to attribute this to postsynaptic inhibitory processes, even recurrent inhibition (Pierrot-Deseilligny and Burke, 2012). There is good evidence that presynaptic inhibition contributes to D1 (Mizuno et al., 1971; El-Tohamy and Sedgwick, 1983; Iles and Roberts, 1987; Iles, 1996; Faist et al., 1996; Capaday et al., 1995; Aymard et al., 2000; *see* Pierrot-Deseilligny and Burke 2012). In Figures 6 and 7, D1 was significantly reduced in patients with MND, and this suggests a decrease in presynaptic inhibition.

Pathology and presynaptic inhibition. Presynaptic inhibition of Ia afferents directed to soleus motoneurones is decreased in patients with spinal cord lesions due to trauma (Faist et al., 1994; Kagamihara and Masakado, 2005) or multiple sclerosis (Nielsen et al., 1995), whether tested using D1, brief vibration of the tendon of tibialis anterior, or the heteronymous facilitation of the soleus H reflex by group Ia afferents in the femoral nerve (for details of these techniques, *see* Methods and Pierrot-Deseilligny and Burke, 2012). There is scant evidence regarding presynaptic inhibition in ALS/MND. Schieppati et al. (1985) reported that there was less inhibition of the soleus H reflex when patients with ALS abruptly relaxed a voluntary contraction of soleus, a finding that they interpreted as absence of activation of presynaptic inhibitory mechanisms. Based on studies using the inhibition produced by brief trains of vibration to the tendon of tibialis anterior, Pierrot-Deseilligny (1990) reported that there is decreased presynaptic inhibition of group Ia afferents projecting to soleus motoneurones in ALS

patients, all of whom apparently had signs of spasticity, unlike the present cohort. Regrettably, the data have not been replicated in a peer-reviewed journal publication, and our findings raises the question whether the spasticity was a relevant association (*see* later).

It is now appreciated that the degenerative process of MND is not always confined to upper and lower motoneurones, and astrocytes may be involved in the pathogenesis of familial ALS, at least in animal models (Bruijn et al. 1997). One explanation for the data in Figures 6 and 7 would be a smaller or less effective Ia afferent volley entering the spinal cord (Vaughan et al., 2015). In motor neurone disease, the "*fusimotor innervation [of the muscle spindle] was almost always abnormal*" (Swash and Fox, 1974), but "*the intrafusal muscle fibres were of normal diameter and the bag and chain nuclear aggregations, the primary and sensory endings, and their afferent nerve fibres were also always normal*". The effects of the gamma motoneurone involvement would not be seen at rest (as in the present experiments) but when gamma motoneurones are active, as in a voluntary contraction (Pierrot-Deseilligny and Burke, 2012). A smaller Ia afferent input to the spinal cord would constitute a sensory abnormality, for which there is considerable evidence in MND, even when sensory loss is not apparent clinically (*e.g.*, Radtke et al., 1986; Heads et al., 1991; Gregory et al., 1993; Mondelli et al., 1993; Isaacs et al., 2006; Hammad et al., 2007; Pugdahl et al., 2007; Cohen-Adad et al., 2013; Iglesias et al., 2015; Isak et al., 2016, 2017; Matamala et al., 2018a). However, one might then expect all Ia inhibitory responses to be less, not just D1, and we favour the alternative explanation, that the decrease in D1 seen here does reflect a change in presynaptic inhibition. This conclusion is consistent with the findings of Pierrot-Deseilligny (1990).

In MND, interneurones are lost at spinal levels "*to a similar extent and in parallel with motor neurons*" (Stephens et al., 2006). The question then arises whether the loss of presynaptic inhibition is due to an interneuronopathy affecting the presynaptic inhibitory interneurone or from abnormal control of that interneurone. The present data do not allow us to distinguish between these possibilities. However corticospinal drives suppress presynaptic inhibitory interneurones directed to

soleus Ia afferent terminals (*see* Hultborn et al., 1987; Meunier and Pierrot-Deseilligny, 1989). One would not expect a decrease in presynaptic inhibition due to a loss of these drives. On the other hand, there is an increasing focus on an interneuronopathy in MND (Chang and Martin, 2009; Hossaini et al., 2011; Martin and Chang, 2012; Turner and Kiernan, 2012; Medelin et al., 2016), and this remains a feasible explanation.

If a defect in presynaptic inhibition of Ia afferents is a generalised phenomenon, one might expect tendon jerk hyperreflexia, regardless of the presence or absence of other upper motoneurone signs. Indeed, in MND tendon jerks are commonly brisk in muscles that are not weak, tone may not be increased, and the Babinski sign is often absent (Swash, 2012; Swash et al., 2020). The absence of other signs is often attributed to the concurrent lower motoneurone degeneration. However, if hyperreflexia can result from an interneuronopathy involving the presynaptic inhibitory interneurone (Turner and Kiernan, 2012), it is challenging to speculate that perhaps hyperactive tendon jerks are not really evidence of corticospinal damage. Perhaps the hyperreflexia in MND represents a spinal pre-motoneuronal abnormality rather than a corticospinal abnormality.

CONFLICT OF INTEREST

None of the authors report conflicts of interest.

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LEGENDS FOR FIGURES

Figure 1: Flowchart for experiments performed on healthy control subjects.

Figure 2: Threshold tracking the soleus H reflex. The unconditioned test stimulus (upward stimulus artefact) produced a well-formed H reflex without an M wave (black traces). The red traces illustrate the reflex responses when a conditioning stimulus 1.4 times motor threshold was delivered to the common peroneal nerve (downward stimulus artefact) 3.5 ms before the test stimulus. The small EMG potential seen immediately after the test stimulus was far-field EMG from the pretibial flexors due to the conditioning stimulus. In the blue traces, the conditioning-test interval was 21 ms, and the intensity of the conditioning stimulus was 1.2 times motor threshold, hence the smaller far-field EMG produced in the pretibial flexors. In the green traces, the conditioning-test interval was 200 ms so the conditioning stimulus is not visible. In all traces, the measurement window was set around the reflex potential, and target amplitude (20% M_{max}) is indicated by the gap between the grey interrupted lines. When the peak-to-peak amplitude of the reflex response exceeded the window, the strength of the next stimulus was reduced. When it was less than the target size, the stimulus was increased.

Figure 3: Recovery cycles of the soleus H reflex following a conditioning stimulus measured using two different methods. Single conditioning stimulus to the innervation of the pretibial flexors, at 1.4 xMT up to 6 ms, and 1.2 xMT thereafter. Conventional constant-stimulus method (upper trace) and threshold tracking (lower trace).) Data are shown as mean (SEM) for 17 subjects. The three inhibitory phases (reciprocal Ia inhibition, D1 and D2) are labelled. The first inhibitory phase is clearer with threshold tracking.

Figure 4: The time taken for measurements using the constant-stimulus method (horizontal axis) and threshold tracking (vertical axis), with the line of identity shown by the interrupted line. Data for healthy subjects (closed symbols) and patients (open symbols). There was no difference in time for the patients and controls. With all but one recording of D2 (healthy subject, the green symbol above the line of identity), the time taken was much less (60%) with threshold tracking. The greater variability of the threshold-tracking responses reflects the gradual tracking to the target level. This contrasts with the constant-stimulus method where changes are instantaneous and the variability reflects the intrinsic variability of the reflex response.

Figure 5: Effect of conditioning stimuli on the recruitment curves for the H reflex and M wave. Left-hand panels: data for 4 healthy subjects, using the constant-stimulus protocol. The curves are: black: unconditioned; red: conditioned, with interval 3.5 ms (reciprocal Ia inhibition); blue: conditioned, 21 ms interval (D1); green: conditioned, 200 ms interval (D2). The inhibitory effects are small, largely confined to the rising phase of the H reflex recruitment curve and produce a slight delay and diminution of H_{max}. Note the difference in time base for the upper and lower panels. Right-hand panels: cumulative sums of the differences between the conditioned and unconditioned traces for data in the left-hand panels. The red traces are the difference between the red (conditioned) and black (unconditioned) recruitment curves shown on the left, and represent reciprocal Ia inhibition. Similarly the blue traces represent D1 and the green D2. Note that, in each subject, the three inhibitory effects begin with the first-recruited motoneurones, ~0.6 xMT for the upper two subjects who had large H_{max}/M_{max} ratios. For the lower two subjects, the higher thresholds are presumably because a greater Ia afferent volley was required to produce the H reflex.

Figure 6: Recovery cycles of the soleus H reflex in control subjects and patients with MND. Single conditioning stimulus. Effects measured using threshold tracking. Mean data for 17 healthy control subjects (filled symbols) and 11 patients with motor neurone disease (open symbols), ± SEM.

Conditioning: single stimulus to the innervation of the pretibial flexors, at 1.4 xMT up to 6 ms and 1.2 xMT thereafter. The extent of each inhibitory phase in Fig. 6 was measured over the interstimulus intervals indicated by the horizontal bars – red (reciprocal Ia), green (D1), blue (D2). The difference in D1 was significant (P = 0.006) but the differences in reciprocal Ia inhibition and D2 were not (*see* also Fig. 7).

Figure 7: The inhibitory phases in controls and patients with MND. Left panel, data from threshold tracking for the 17 control subjects (filled symbols) and 11 patients (open symbols), with values for different subjects sometimes superimposed. The areas of the inhibitory phases were measured over 3-4 ms for reciprocal Ia inhibition (red), 11-21 ms for D1 (blue) and 100-300 ms for D2 (green). The difference in D1 was significant (P = 0.006). In the right panel (black), the ages of the patients (open symbols) overlap those of the controls (filled symbols), but the mean difference is significant (P = 0.016). In each data set in both panels, the mean is shown by the horizontal black line.

Figure 1

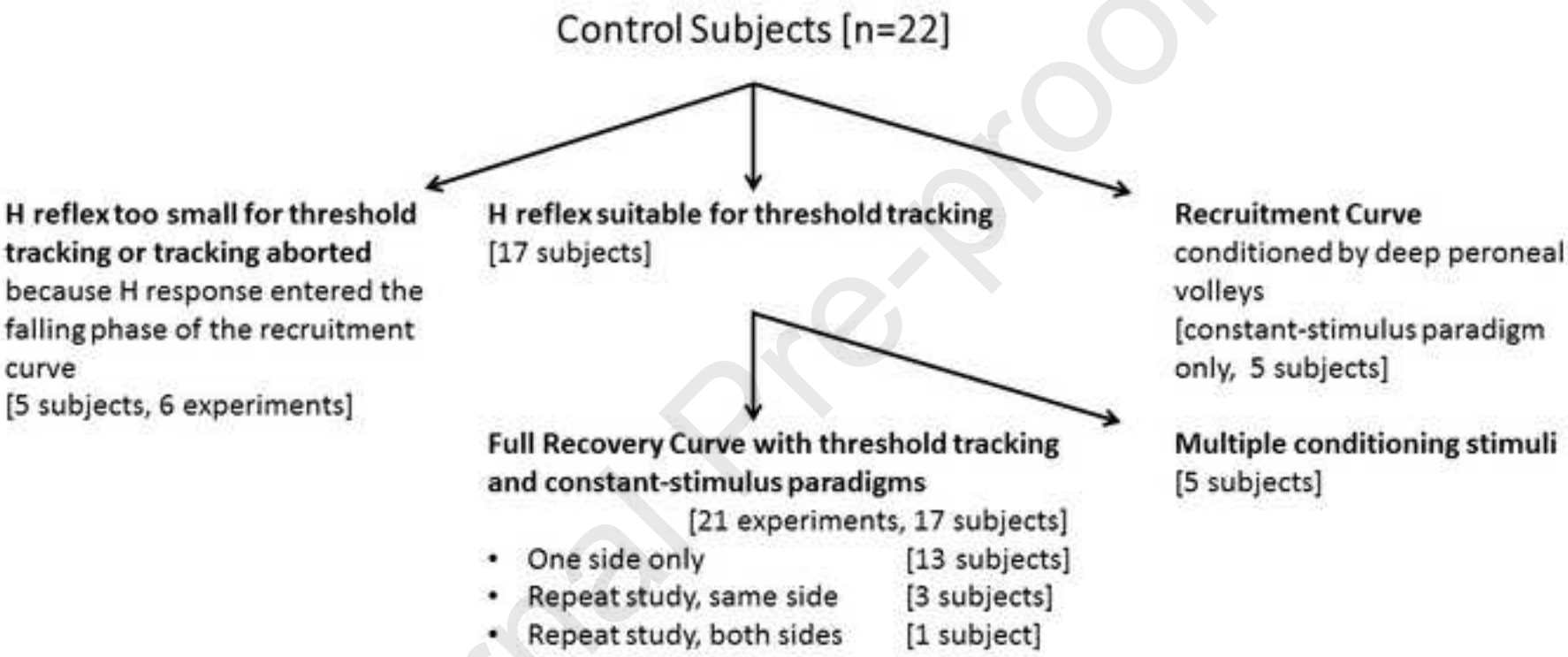

Figure 2

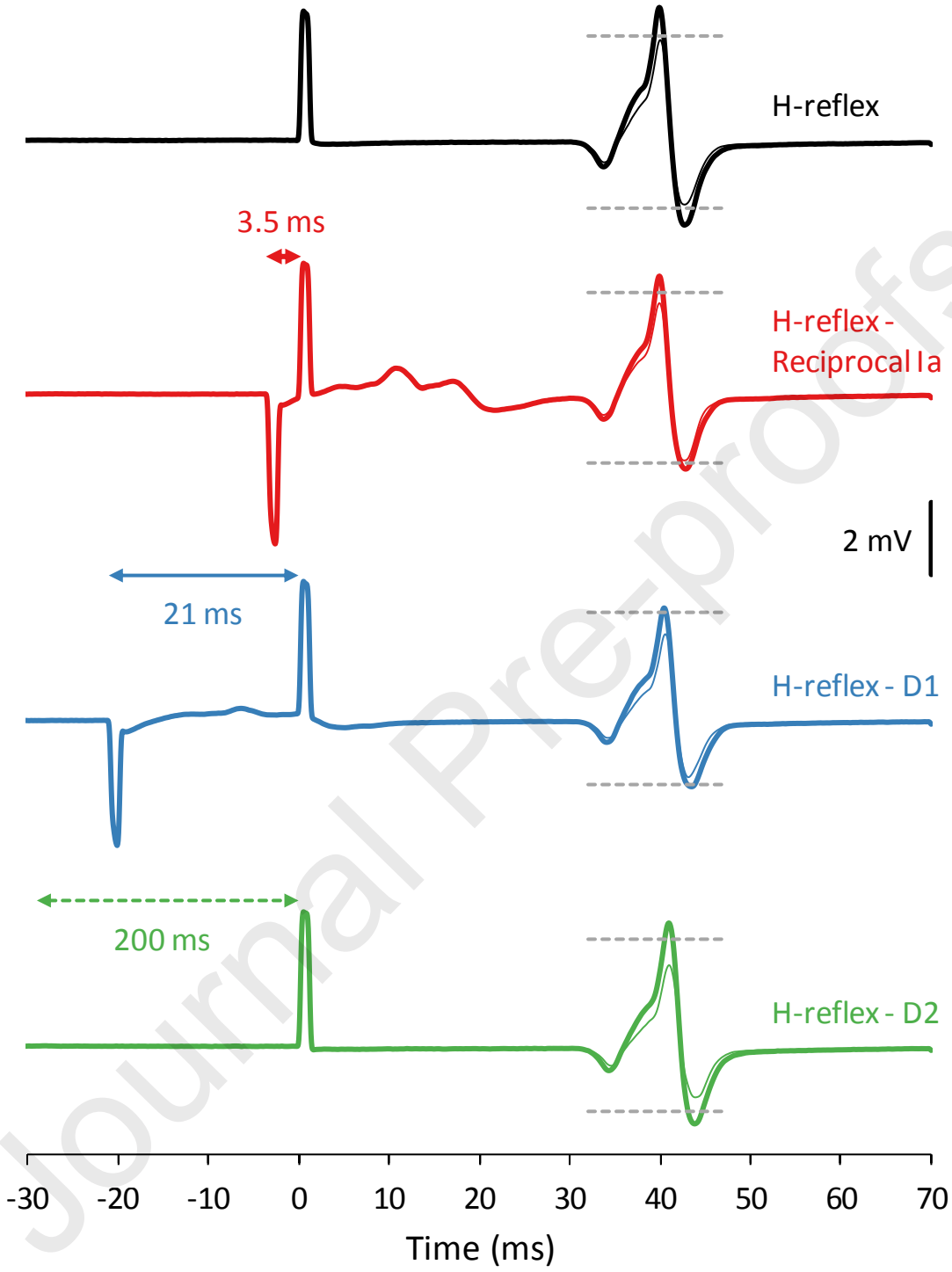

Figure 3

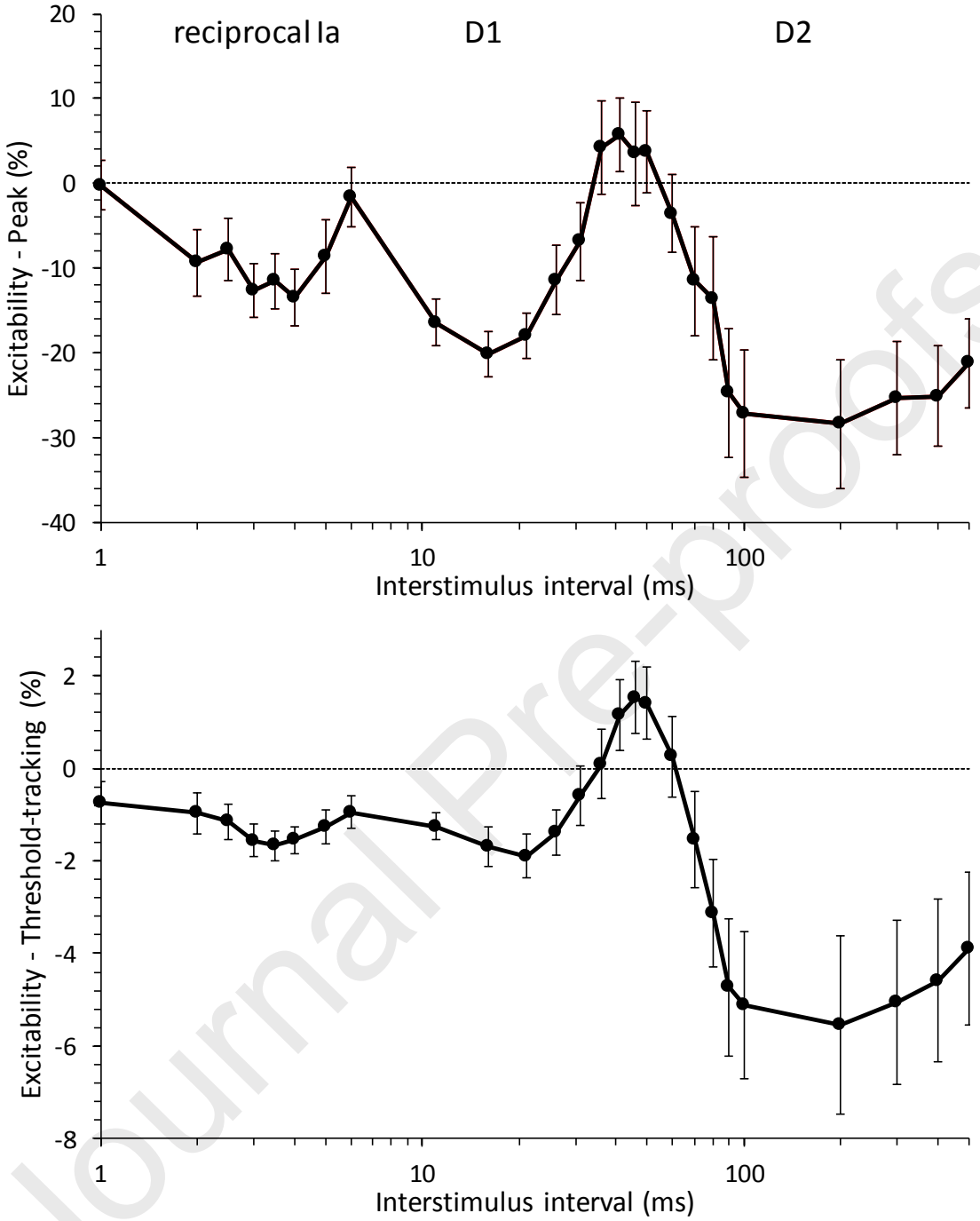

Figure 4

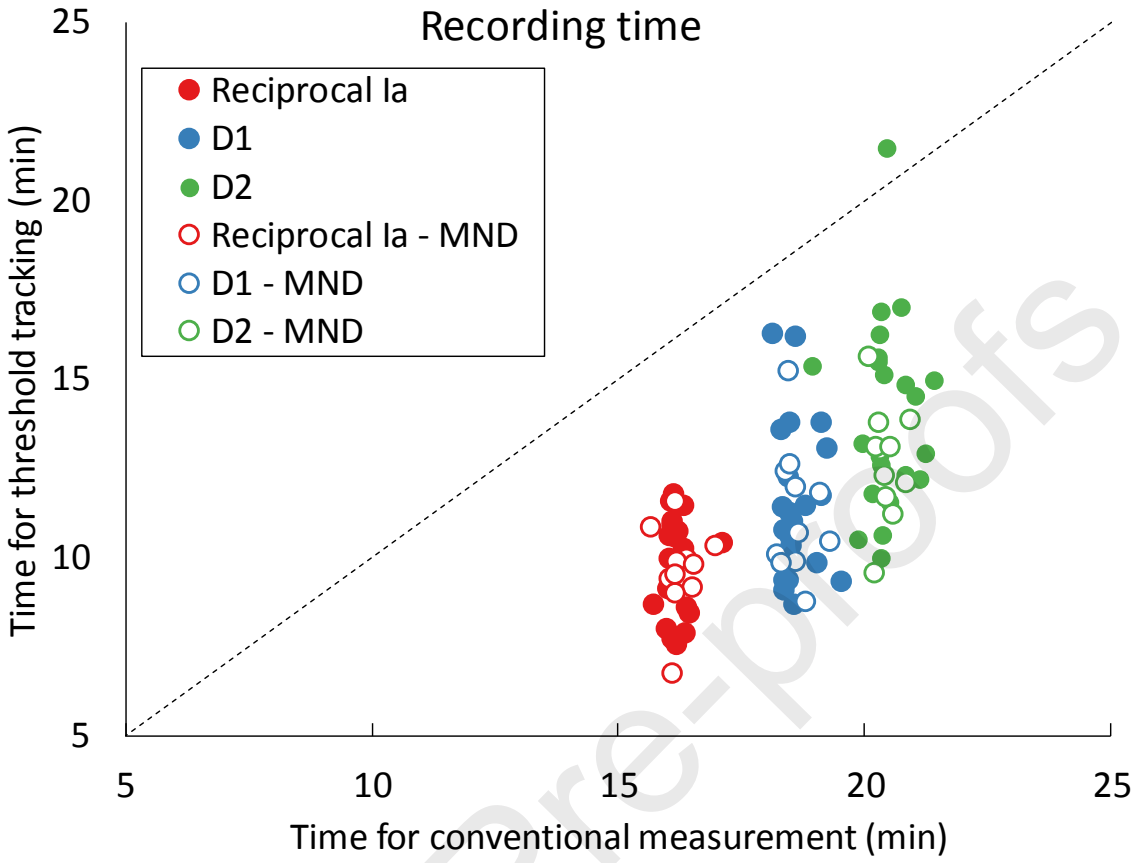

Figure 5

Figure 5

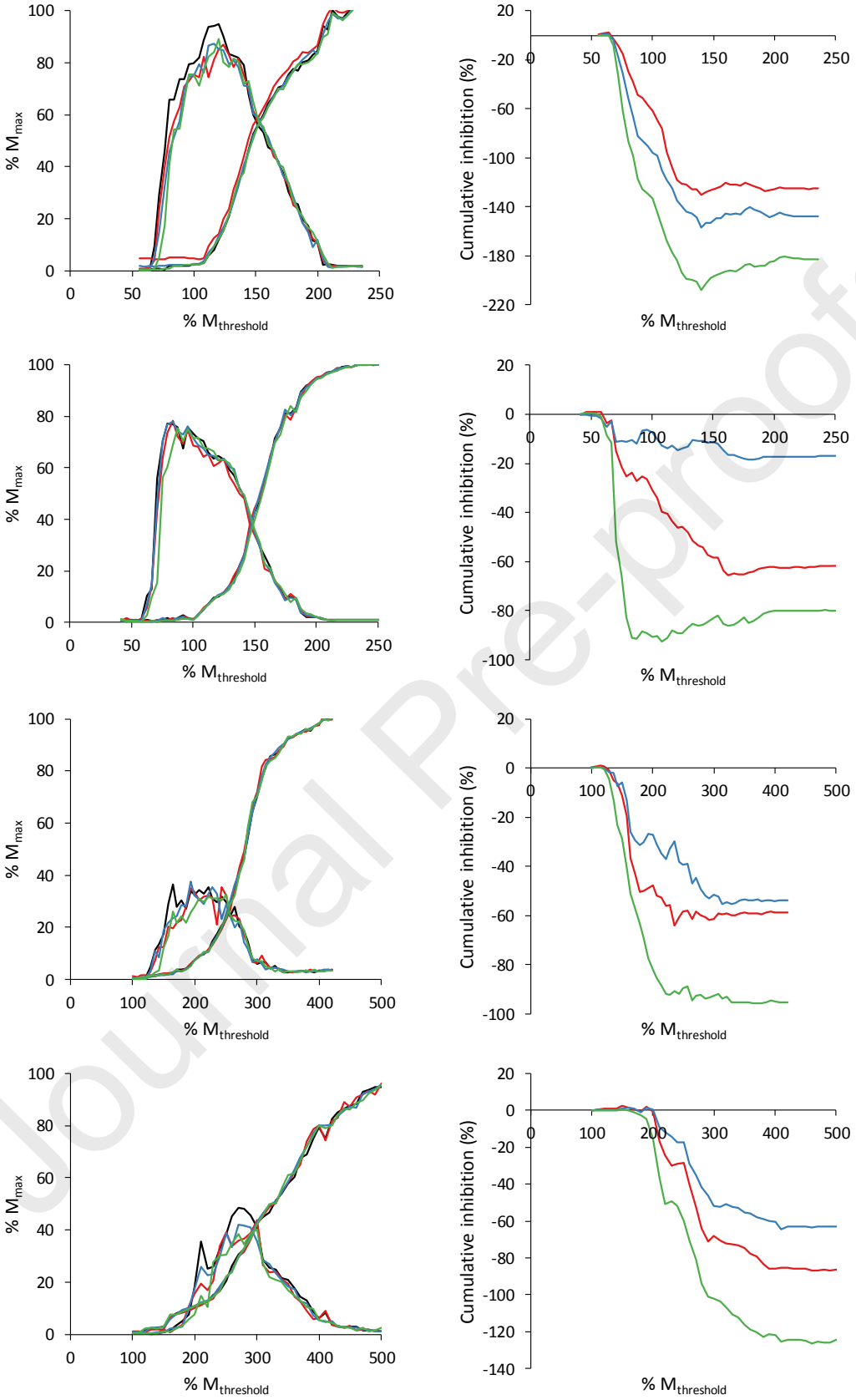

Figure 6

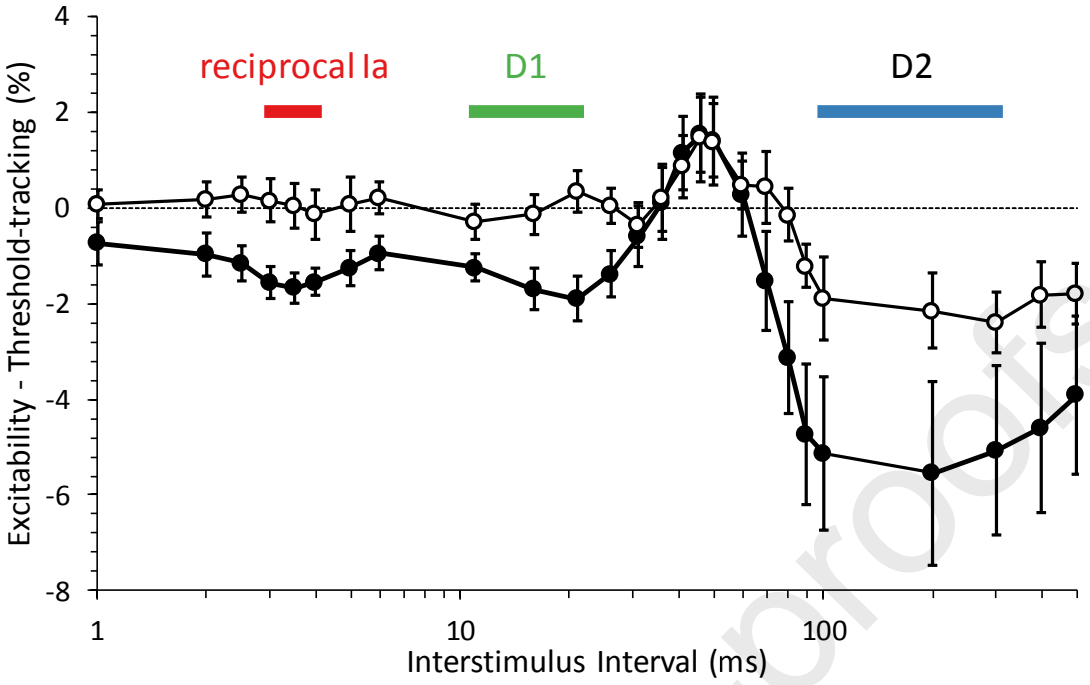

Figure 7

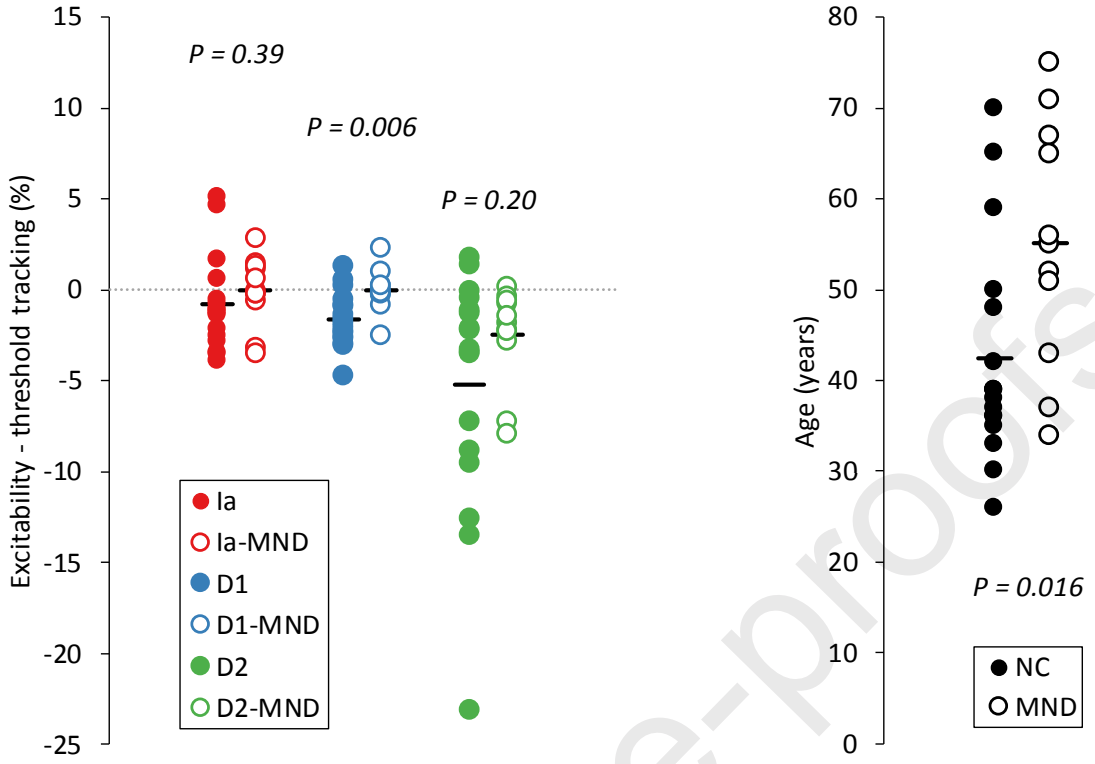

Table 1: Clinical features

Patient	Age (years)	Sex	Diagnostic certainty (Awaji criteria)	Site of onset	Disease duration (months)	UMN score (max 16)	LL UMN signs	ALSFRS-R (max 48)	MRC LL score (normal 30)	MRC TA score (normal 5)
1	67	F	PLS	UL	120	16	Present	40	30	5
2	65	M	Definite	LL	38	7	Present	28	19	3
3	52	M	Possible *	Bulbar	12	12	Present	46	30	5
4	75	M	Definite	UL	18	14	Present	37	30	5
5	71	M	Probable (LMN mainly) **	UL	62	0	Absent	41	26	4
6	37	M	Definite	UL	18	6	Absent	40	24	4
7	43	M	Flail arm, Probable	UL	30	8	Absent	42	30	5
8	51	M	Definite	Bulbar	25	15	Present	26	23	4
9	34	M	Definite	UL	6	13	Present	45	30	5
10	55	M	Definite	Bulbar	46	16	Present	43	29	5
11	56	M	Definite	LL	10	13	Present	45	26	5

ALSFRS-R = revised ALS Functional Rating Scale (Cedarbaum et al. 1999).
Upper motor neuron (UMN) score as in Iwata et al. (2008).
Medical Research Council (MRC) LL score = sum of MRC strength scores for hip flexion, knee extension and ankle dorsiflexion bilaterally (see Kleyweg et al. 1991).
*,** = patients died of ALS 45 and 27 months after testing, respectively.