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Research paper

The test–retest reliability of large and small fiber nerve excitability testing with threshold tracking

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ABSTRACT

Objective: Standard nerve excitability testing (NET) predominantly assesses Aα- and Aβ-fiber function, but a method examining small afferents would be of great interest in pain studies. Here, we examined the properties of a novel perception threshold tracking (PTT) method that preferentially activates Aδ-fibers using weak currents delivered by a novel multipin electrode and compared its reliability with NET.
Methods: Eighteen healthy subjects (mean age:34.06 ± 2.0) were examined three times with motor and sensory NET and PTT in morning and afternoon sessions on the same day (intra-day reliability) and after a week (inter-day reliability). NET was performed on the median nerve, while PTT stimuli were delivered through a multipin electrode located on the forearm. During PTT, subjects indicated stimulus perception via a button press and the intensity of the current was automatically increased or decreased accordingly by Qtrac software. This allowed changes in the perception threshold to be tracked during strength-duration time constant (SDTC) and threshold electrotonus protocols.
Results: The coefficient of variation (CoV) and interclass coefficient of variation (ICC) showed good–excellent reliability for most NET parameters. PTT showed poor reliability for both SDTC and threshold electrotonus parameters. There was a significant correlation between large (sensory NET) and small (PTT) fiber SDTC when all sessions were pooled ($r = 0.29$, $p = 0.03$).
Conclusions: Threshold tracking technique can be applied directly to small fibers via a psychophysical readout, but with the current technique, the reliability is poor.
Significance: Further studies are needed to examine whether Aβ-fiber SDTC may be a surrogate biomarker for peripheral nociceptive signalling.

1. Introduction

Peripheral nerve excitability testing (NET) performed using the threshold-tracking technique can be used to infer changes in the membrane potential of sensory and/or motor fibres underlying the site of stimulation (Bostock et al., 1998; Kiernan and Bostock, 2000). Through the use of standardised protocols (e.g. TROND), multiple biophysical properties belonging to these peripheral fibres can be assessed, with the specific profile of changes in these variables able to provide insights into the molecular mechanisms (e.g. modulation of ion channel or ionic pump activity) contributing to clinical or preclinical phenotypes (Bostock et al., 1998; Krarup and Moldovan, 2009; Kiernan et al., 2020; Kristensen

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et al., 2021; Kroigard et al., 2022). Furthermore, the same properties can also be used as pharmacodynamic biomarkers to study in vivo target engagement, thereby facilitating the development of novel medications. One such disease area where this may be beneficial is in the management of chronic pain, where NET has implicated dysfunction of axonal ion channels such as voltage-gated sodium channels as contributory factors to the hyperexcitability observed in painful neuropathies (Moldovan et al., 2013; Farrar et al., 2017; Tigerholm et al., 2020). Typically, these studies of nerve excitability have been performed on either large sensory fibres – where the response is mediated by Aβ fibers – or on motor fibres (Kiernan et al., 2020). Conversely, the function of Aδ and C fibers are of greater relevance from the pain perspective (Verdugo et al., 2022). Approaches such as quantitative sensory testing (Isak et al., 2021), laser and contact heat evoked potentials (De Schoenmacker et al., 2021; Isak et al., 2021), and skin biopsies (Gylfadottir et al., 2021) are the most widely utilised methods for evaluating the integrity and structural changes of these fibres in humans, however, they cannot assess specific changes in ion-channel function. Microneurography can evaluate the functional properties of C-fibers but is a time-consuming technique managed in only few centers in the World (Verdugo et al., 2022).

The perception threshold tracking (PTT) technique is a novel application of the conventional NET protocol that examines the function of a population of fibers presumed to include a significant proportion of thinly-myelinated Aδ fibers with nerve terminals in the epidermis that are activated by weak currents delivered using multipin electrodes. Preferential activation of small cutaneous nerve fibers by electrical stimulation through an array of small area pin cathodes has previously been shown (Notermans, 1966; Bromm and Meier, 1984; Klein et al., 2004; Mouraux and Iannetti, 2018).

In this study, we aimed to investigate the test–retest reliability of large and small fiber nerve excitability testing with threshold tracking, and to correlate the measures obtained across fibre classes.

2. Methods

This study was designed on the basis of IMI2-PainCare-BioPain-RCT1, which is a multicenter clinical trial (Nochi et al., 2022). In addition to large fiber NET variables as primary endpoints, the small fiber PTT is a secondary endpoint measure in the IMI2-PainCare-BioPain-RCT1 trial.

The present study is a cross-sectional study with inclusion of healthy participants from March 2020 to December 2020 and after a stop due to COVID-19 restrictions again from December 2021 to January 2022.

2.1. Participants

Eighteen healthy individuals aged ≥ 18 years (6 women and 12 men) participated in the study at the Department of Clinical Neurophysiology, Aarhus University Hospital. Healthy volunteers were recruited from announcements. Exclusion criteria were any neurological disorders or medication for any neurological or psychiatric disease including painkillers. The study protocol was approved by the regional scientific ethics committee (no. 1-10-72-133-19) and the study was registered in the internal registration to the Danish Data Protection Agency of the Central Denmark Region (no. 787443). All participants gave their written consent prior to the study.

All participants were examined with motor and sensory NET and PTT during separate morning and afternoon sessions on the same day (intra-day reliability) and once again after at least a week (inter-day reliability). Each session took approximately 1 h (½ hour for motor and sensory NET and ½ hour for PTT).

Skin temperature was maintained between 32 °C and 34 °C during the recordings.

2.2. Equipment

The stimulation and recording were controlled by QtracS (written by H. Bostock, Copyright Institute of Neurology, University College London, UK). The excitability setup consists of a DS5 bipolar stimulator, a D440 amplifier and a HumBug noise eliminator (Digitimer Ltd, UK), and a Data Acquisition Device (USB-6221-BNC, National Instruments, USA).

2.3. Nerve excitability testing (NET)

In this study, stimulation was administered through the use of two electrodes positioned over the median nerve on the right side with the cathode located at the wrist and the anode placed proximally on the forearm (Fig. 1). Motor NET involved the placement of a surface recording electrode and a reference electrode above the abductor pollicis brevis muscle and the metacarpophalangeal joint respectively. Meanwhile, sensory NET required the placement of two recording electrodes over the inter-phalangeal joints of the index finger. The study utilized TRONDOLM (motor) and TRONDOLS (sensory) protocols available through the QtracW software. These protocols enabled assessment of stimulus response curve, strength duration relationship, threshold electrotonus and recovery cycle of each fibre type. Notably, these protocols differ from the traditional TROND protocols in that they offer greater efficiency since they are faster. During strength–duration property testing, fewer stimuli are applied, and the 0.3-millisecond duration test stimulus is excluded. Furthermore, the threshold electrotonus testing forgoes the 20% hyperpolarizing conditioning stimulus in a favor of a 70% hyperpolarizing conditioning stimulus. It is also worth noting that the current–threshold relationship is not included in either the TRONDOLM or TRONDOLS protocols.

These protocols have been used in a few recent studies (Bennedsgaard et al., 2020a, 2020b; Kristensen et al., 2021: Themistocleous et al., 2022) but their reliability has yet to be assessed.

A more comprehensive description of the nerve excitability testing methods employed in this study can be found in previously published literature (Kristensen et al., 2021; Themistocleous et al., 2022). The international guidelines have been followed for the methodology of all sensory and motor excitability tests (Kiernan et al., 2020).

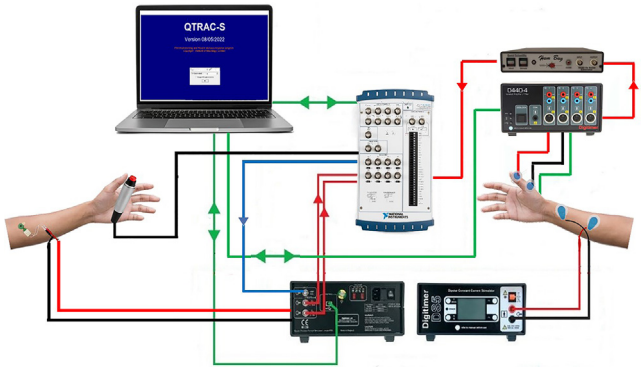

Fig. 1. Illustration of motor and sensory nerve excitability testing and perception threshold tracking. The equipment set-up and the placement of stimulation and recording electrodes are shown.

In brief, the supramaximal response amplitude is initially identified, and subsequently the stimulus is automatically reduced until no further activation is observed. The stimulus current that evokes a fixed fraction of the maximum response (specifically, 40% for CMAP and 50% for SNAP) is then defined as the threshold, and the corresponding response is tracked throughout the duration of the recording. This methodology yields a stimulus response curve, which is subsequently utilized in further analyses.

The strength-duration relationship entails the examination of the stimulus strength necessary to achieve the target response at four distinct, predetermined stimulus durations. The evaluation of the strength-duration time constant (SDTC) and rheobase, as outlined by Weiss' law, serve as the key parameters for this analysis. Specifically, rheobase is determined by the slope of the graph generated through linear regression, while the SDTC corresponds to the x-intercept of this function.

The threshold electrotonus technique employs a subthreshold polarizing current of long duration as the conditioning stimulus to modify the potential of the internodal membrane, thereby facilitating an inquiry into internodal properties. The methodology involves the measurement of the threshold potential prior to, during, and after the administration of the polarizing current. Specifically, depolarizing and hyperpolarizing conditioning pulses of 20% and 40% magnitude are administered for a duration of 100 ms, while a 70% hyperpolarizing electrotonus is applied for a duration of 200 ms.

Recovery cycle uses a supramaximal conditioning stimulus prior to the test stimulus. Refractoriness is normally followed by phases of superexcitability and late subexcitability, before returning to unconditioned excitability.

2.4. Perception threshold tracking (PTT)

PTT was performed using the TRONDRT4B protocol, part of the QTracW software. This protocol differs from NET by having a higher detection accuracy of SDTC with seven different predetermined stimulus durations, and it does not include recovery cycle. A novel multi-pin electrode (EPS-P10, MRC Systems GmbH, Heidelberg) was placed approximately 5 cm below the cubital fossa area on the right forearm. The electrode consisted of 10 tungsten pins (0.25 mm diameter) arranged in a circle (5 mm diameter) which together served as the cathode, and a tin-plated metallic surface (area 24x20 mm^2) acting as the anode. The distance between the center of the cathode pins and the anode was 20 mm. The cathode and anode were fixed to the skin with insulating adhesive rings and conductive gel pads respectively. The gel pads are similar to the ones used for TENS electrodes. Perception threshold was assessed by increasing the intensity of repeated stimuli until the participant felt the stimulus, which was indicated by pressing a button (Fig. 1). The stimulus intensity was then automatically adjusted up/down every 2 s using Qtrac software so that changes in perceptual threshold could be monitored during the SDTC and threshold electrotonus protocols.

2.5. Data analysis

Statistical analysis was performed using QtracP and Excel, and the figures were generated using QtracP. Unless otherwise indicated, group values are given by means ± standard deviation of the mean (SD). Normal distribution was determined based on Lilliefors test, and if the data were not normally distributed, calculations of significance for differences in medians were performed by non-parametric tests. P values <0.05 were considered statistically significant.

The sessions were compared using repeated measures ANOVA. Sensory NET and PTT values for SDTC was correlated using linear regression analyses.

The intra- and inter-day reliability was tested by coefficient of variation (CoV) as defined by the ratio of the standard deviation to the mean of the NET and PTT values. The interpretation of CoV was as, CoV ≤ 10%: excellent, CoV = 10–20%: good, CoV = 20–30%: acceptable, and CoV > 30: poor. Additionally, the test-restest reliability were assessed using inter-class correlation coefficients (ICC). The interpretation of ICC was as, ICC > 0.90: excellent, ICC = 0.75–0.90: good, ICC = 0.50–0.75: moderate and ICC < 0.50: poor.

3. Results

All 18 participants (mean age: 34.06 ± 2.0, range: 19–49, 6 women and 12 men) completed each NET and PTT session and tolerated the examinations well. The motor and sensory NET and PTT for SDTC and threshold electrotonus for 3 sessions are shown in Fig. 2.

3.1. Intra- and inter-day reliability of NET and PTT

The mean NET and PTT values, and CoVs and ICCs for each parameter are summarized in Tables 1–3. The mean values did not differ between sessions apart from S3 in motor NET (p = 0.032) (Table 1), TEd(10–20 ms) (p = 0.041) and refractoriness (0.047) in sensory NET (Table 2) and TEh(10–20 ms) (p = 0.008) in PTT (Table 3).

Among the motor NET variables, the intra- and inter-day reliabilities were excellent or good for most parameters. The only variables with poor reliability either using CoV or ICC were rheobase, TEh(20–40 ms), relative refractory period, refractoriness at 2.5 m and S3 (Table 1).

The sensory NET variables showed a similar pattern with excellent or good reliabilities for most parameters. The variables with poor reliability using CoV or ICC were accommodation half-time (ms), TEd20(10–20 ms), TEd20 (peak), and S3 (−70%), TEd20(10–20 ms) and refractoriness at 2.5 m Table 2).

For PTT, all strength-duration relationship and threshold electrotonus parameters showed poor reliability both intra-day and inter-day using CoV or ICC (Table 3).

3.2. Correlation between large and small fiber excitability parameters

There was a significant correlation in SDTC between the sensory NET and PTT when all sessions are combined (r = 0.028, p = 0.03) while for individual sessions the correlation was not significant (Day 1 morning, r = 0.34, p = 0.169, Day 1 afternoon, r = 0.25, p = 0.325 and Day 2 (r = 0.46, p = 0.055) (Fig. 3).

4. Discussion

We found excellent test–retest reliability for large fiber sensory and motor NET for most variables whereas PTT showed poor reliability for all parameters. There was a significant but poor correlation between large and small sensory fiber SDTC.

High test–retest reliability of the TROND protocol is well-recognised, however it has never been tested systematically. In 2017, shorter versions of the motor (TRONDOLM) and sensory (TRONDOLS) TROND protocols were introduced for clinical and research settings that the time ensured for the excitability testing experiments might be limited. These shorter protocols were used in a few recent studies (Bennedsgaard et al., 2020a, 2020b; Kristensen et al., 2021; Themistocleous et al., 2022). However, like the traditional TROND protocols, the test–retest reliability of these

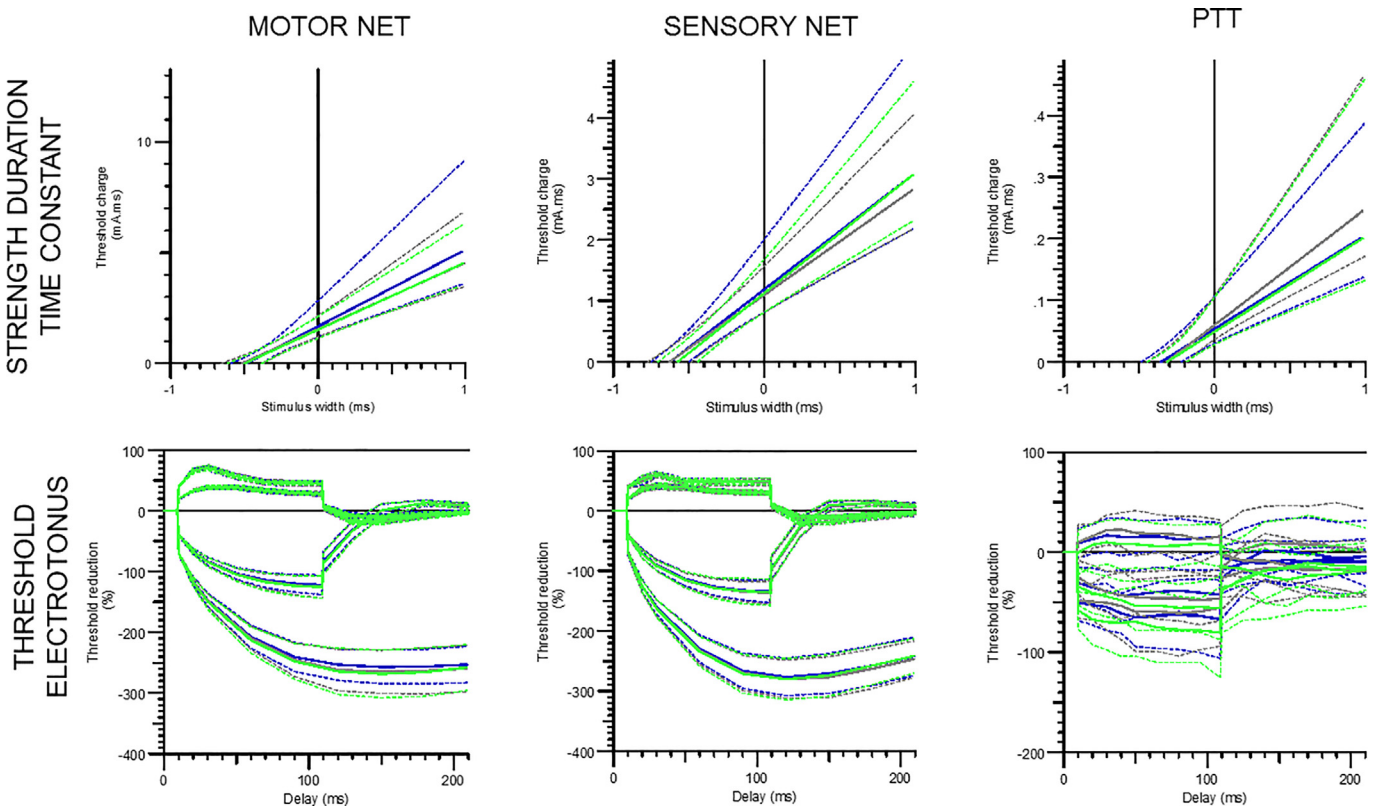

Fig. 2. The strength-duration-time-constant (SDTC) and threshold electrotonus waveforms are shown for 3 sessions in motor and sensory nerve excitability testing, and perception threshold tracking (PTT). Dotted lines indicate standard deviation of mean. Blue lines indicate Day 1, morning session, gray lines Day 1, afternoon and green lines indicate Day 2 examinations. Top row panels are stimulus charge versus duration plots, so rheobase is the slope and SDTC is the X intercept. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
The intra-day and inter-day test–retest reliability for motor nerve excitability tests (NET) depicted as coefficient of variation (CoV), interclass correlation coefficient (ICC) and mean values for all three sessions.

	Variable Name	CoV (%)		ICC		Mean		
		Intra-day	Inter-day	Intra-day	Inter-day	Day 1 Morning *Value ± SD*	Day 1 Afternoon *Value ± SD*	Day 2 *Value ± SD*
Strength duration relationship	Strength-duration time constant (ms)	6.7	7.1	0.86	0.82	0.51 ± 0.11	0.55 ± 0.15	0.51 ± 0.12
	Rheobase (mA)	15.1	18.6	**0.33**	**0.18**	3.46 ± 1.89	3.00 ± 1.17	3.05 ± 0.89
Threshold electrotonus	TEh(90–100 ms)	3.7	4.6	0.87	0.82	−121.64 ± 15.72	−125.24 ± 17.46	−125.21 ± 17.85
	TEd(10–20 ms)	1.3	2.3	0.91	0.78	68.39 ± 4.09	69.08 ± 4.08	68.74 ± 4.3
	TEd(40–60 ms)	1.9	4.0	0.87	0.51	51.71 ± 4.14	51.93 ± 4.26	51.64 ± 3.23
	TEd(90–100 ms)	2.0	4.4	0.90	0.68	45.14 ± 4.41	45.28 ± 4.69	45.96 ± 3.36
	TEh(10–20 ms)	2.0	2.5	0.86	0.86	−74.94 ± 5.9	−75.64 ± 5.68	−75.93 ± 6.26
	TEd(undershoot)	11.8	11.0	0.53	0.68	−16.75 ± 3.67	−18.69 ± 3.55	−18.04 ± 4.23
	TEh(overshoot)	11.4	18.1	0.83	0.57	12.75 ± 4.19	12.62 ± 3.43	12.88 ± 3.3
	TEd(peak)	1.3	2.5	0.90	0.73	67.82 ± 3.82	68.36 ± 3.93	68.13 ± 3.94
	S2 accommodation	3.7	6.0	0.87	0.82	22.68 ± 2.84	23.08 ± 3.58	22.17 ± 3.77
	Accommodation half-time (ms)	3.6	4.8	0.77	0.73	41.11 ± 5.17	40.87 ± 4.85	40.20 ± 4.91
	TEh(20–40 ms)	2.3	2.7	0.57	**0.43**	−93.35 ± 8.31	−94.42 ± 8.05	−94.89 ± 9.24
	TEh(slope 101–140 ms)	6.2	5.2	0.88	0.85	2.05 ± 0.31	2.15 ± 0.36	2.16 ± 0.33
	TEd20(peak)	2.6	3.5	0.74	0.78	38.32 ± 2.77	39.23 ± 2.89	39.15 ± 3.79
	TEd40(Accom)	3.6	5.8	0.86	0.81	22.78 ± 2.79	23.21 ± 3.24	22.52 ± 3.69
	TEh(peak −70%)	3.5	3.9	0.88	0.86	−259.04 ± 27.68	−266.82 ± 36.15	−269.51 ± 39.33
	S3(-70%)	**62.7**	**74.9**	**0.43**	**0.48**	**4.97 ± 5.58**	**6.06 ± 6.31**	**9.02 ± 7.3**
	TEd20(10–20 ms)	2.8	2.9	0.67	0.74	35.32 ± 1.97	36.37 ± 2.58	36.08 ± 2.93
Recovery cycle	RRP (ms)	3.5	4.9	0.66	**0.29**	3.06 ± 0.27	3.05 ± 0.23	3.02 ± 0.23
	Superexcitability (%)	4.0	8.9	0.96	0.82	−25.03 ± 6.66	−25.47 ± 6.58	−25.24 ± 6.27
	Subexcitability (%)	7.4	10.3	0.86	0.79	15.00 ± 3.82	14.84 ± 3.8	15.32 ± 3.96
	Refractoriness at 2.5 ms (%)	28.4	**50.4**	0.57	**0.43**	26.68 ± 14.9	28.39 ± 15.48	21.51 ± 13.72
	Superexcitability at 7 ms (%)	3.7	9.3	0.95	0.88	−24.08 ± 6.5	−25.37 ± 5.54	−23.49 ± 6.53
	Superexcitability at 5 ms (%)	6.7	8.8	0.82	0.84	−25.21 ± 7.97	−27.51 ± 5.34	−26.37 ± 6.46

SD = Standard deviation. RRP = Relative refractory period. Bold mean ± SD values indicate significant difference in mean values ($p < 0.05$) between the three sessions using repeated measures ANOVA. Bold CoV and ICC values indicate poor test–retest reliability.

Table 2
The intra-day and inter-day test–retest reliability for sensory nerve excitability tests (NET) depicted as coefficient of variation (CoV), interclass correlation coefficient (ICC) and mean values for all three sessions.

	Variable Name	CoV (%)		ICC		Mean		
		Intra-day	Inter-day	Intra-day	Inter-day	Day 1 Morning *Value ± SD*	Day 1 Afternoon *Value ± SD*	Day 2 *Value ± SD*
Strength duration relationship	Strength-duration\time constant (ms)	9.4	11.4	0.68	0.69	0.63 ± 0.14	0.65 ± 0.14	0.59 ± 0.14
	Rheobase (mA)	13.5	19.7	**0.33**	**0.23**	1.91 ± 0.82	1.75 ± 0.57	1.97 ± 0.71
Threshold electrotonus	TEh(90–100 ms)	4.7	4.8	0.83	0.86	−133.64 ± 18.33	−136.10 ± 18.49	−135.08 ± 21.66
	TEd(10–20 ms)	4.1	4.7	0.58	0.55	**56.50 ± 5.05**	**54.68 ± 5.53**	**57.60 ± 3.67**
	TEd(40–60 ms)	3.3	2.9	0.88	0.88	**49.01 ± 4.65**	**47.64 ± 4.83**	**48.66 ± 4.57**
	TEd(90–100 ms)	3.6	3.3	0.78	0.83	49.37 ± 4.76	48.33 ± 5.55	49.59 ± 5
	TEh(10–20 ms)	3.8	3.2	0.73	0.83	−80.53 ± 8.08	−81.09 ± 7.7	−82.05 ± 7.51
	TEd(undershoot)	8.5	10.3	0.59	0.62	−19.07 ± 3.82	−19.16 ± 3.84	−19.20 ± 4.28
	TEh(overshoot)	14.6	12.0	0.55	0.73	13.77 ± 3.78	14.14 ± 4.36	14.25 ± 5.08
	TEd(peak)	2.5	2.5	0.79	0.82	58.20 ± 3.85	56.70 ± 4.09	58.04 ± 3.47
	S2 accommodation	29.2	22.4	0.67	0.75	8.87 ± 4.63	8.36 ± 3.89	8.45 ± 3.4
	Accommodation half-time (ms)	20.5	21.6	**0.09**	**0.15**	35.11 ± 12.18	35.11 ± 13.14	30.43 ± 8.03
	TEh(20–40 ms)	3.2	3.6	0.85	0.79	−101.83 ± 10.33	−103.21 ± 10.54	−103.55 ± 10.7
	TEh(slope 101–140 ms)	6.1	6.1	0.72	0.71	2.52 ± 0.38	2.58 ± 0.4	2.59 ± 0.43
	TEd20(peak)	5.1	3.9	**0.50**	0.70	40.71 ± 3.94	40.09 ± 4.29	41.26 ± 4.27
	TEd40(Accom)	17.8	15.5	0.75	0.70	10.26 ± 4.28	9.77 ± 2.77	10.07 ± 3.09
	TEh(peak, −70%)	4.1	4.3	0.79	0.75	−276.27 ± 31.2	−280.03 ± 31.78	−279.71 ± 34.22
	S3(-70%)	22.8	18.9	**0.42**	0.85	29.03 ± 13.47	27.83 ± 9.91	32.93 ± 12.83
	TEd20(10–20 ms)	5.2	4.3	**0.49**	0.67	38.68 ± 4.21	37.87 ± 4.19	39.51 ± 4.17
Recovery cycle	RRP (ms)	4.4	6.2	0.90	0.70	3.42 ± 0.54	3.46 ± 0.43	3.35 ± 0.44
	Superexcitability (%)	6.7	7.1	0.87	0.85	−20.69 ± 5.35	−20.61 ± 4.81	−20.77 ± 5.08
	Subexcitability (%)	10.3	12.2	0.70	0.64	10.52 ± 2.2	10.58 ± 2.7	10.54 ± 2.69
	Refractoriness at 2.5 ms (%)	**34.5**	**32.0**	0.71	0.72	**24.79 ± 19.74**	**27.44 ± 15.17**	**19.73 ± 15.23**
	Superexcitability at 7 ms (%)	6.5	9.1	0.86	0.74	−20.73 ± 5.19	−20.71 ± 4.76	−20.44 ± 5.17
	Superexcitability at 5 ms (%)	11.2	12.5	0.84	0.81	−19.80 ± 6.52	−19.41 ± 6.18	−20.23 ± 5.17

SD = Standard deviation, RRP = Relative refractory period. Bold mean ± SD values indicate significant difference in mean values ($p < 0.05$) between the three sessions using repeated measures ANOVA. Bold CoV and ICC values indicate poor test–retest reliability.

Table 3
The intra-day and inter-day test–retest reliability for perception threshold tracking (PTT) depicted as coefficient of variation (CoV), interclass correlation coefficient (ICC) and mean values for all three sessions.

	Variable Name	**CoV (%)**		**ICC**		**Mean**		
		Intra-day	Inter-day	Intra-day	Inter-day	Day 1 Morning *Value ± SD*	Day 1 Afternoon *Value ± SD*	Day 2 *Value ± SD*
Strength duration relationship	Strength-duration\time constant (ms)	22.8	25.8	**0.17**	**0.23**	0.36 ± 0.13	0.34 ± 0.14	0.34 ± 0.14
	Rheobase (mA)	25.6	25.9	0.51	**0.47**	0.15 ± 0.06	0.19 ± 0.08	0.15 ± 0.08
Threshold electrotonus	TEh(90–100 ms)	**97.7**	**97.1**	**0.09**	**0.19**	−66.06 ± 37.65	−56.70 ± 38.27	−79.63 ± 39.23
	TEh(10–20 ms)	**30.9**	**31.5**	0.57	**0.47**	−53.84 ± 20.69	−50.15 ± 21.92	−68.54 ± 26.14
	TEh(overshoot)	**60.3**	**64.7**	**0.04**	**0.06**	18.57 ± 20.64	28.84 ± 28.2	16.21 ± 16.7
	TEh(20–40 ms)	**57.0**	**58.1**	**0.34**	**0.41**	−60.63 ± 21.11	−54.55 ± 32.11	−70.06 ± 30.81
	TEh(slope 101–140 ms)	**36.3**	**40.6**	**0.16**	**0.06**	**0.71 ± 0.52**	**0.57 ± 0.52**	**0.55 ± 0.46**
	TEd20(peak)	**32.2**	**31.0**	**0.04**	**0.37**	24.94 ± 10.36	29.97 ± 11.25	24.09 ± 11.71
	TEd20(10–20 ms)	**132.6**	**49.8**	**0.15**	**0.37**	14.89 ± 15.26	19.02 ± 12.75	8.44 ± 21.11
	TEh20(10–20 ms)	**33.5**	28.4	**0.26**	**0.47**	−37.24 ± 18.83	−36.91 ± 20.17	−42.73 ± 19.55

SD = Standard deviation. Bold mean ± SD values indicate significant difference in mean values ($p < 0.05$) between the three sessions using repeated measures ANOVA. Bold CoV and ICC values indicate poor test–retest reliability.

protocols has never been investigated. We found in this study good reliability for the TRONDOLM (motor) and TRONDOLS (sensory) protocols. The good sensory NET test–retest reliability similar to the motor NET is in contrast to previous concerns for poor reliability of sensory NET due to its smaller signal-to-noise ratio. However, this may still be a challenging issue in disease while tracking very small sensory action potentials in patients.

Although the reliability of TRONDOLM (motor) and TRONDOLS (sensory) protocols were good, we do not know how the reliability compares to the standard TROND protocol, and whether for example the gain in time in these shorter protocols (approximately 4–5 min per nerve) justify the impairment of modeling ability which is the main strength of the standard NET. A head-to-head comparison of the short and standard NET protocols are necessary in healthy and diseased nerves to clarify these points.

We found the highest variability in motor and sensory NET in S3 to 70% hyperpolarizing currents. Hyperpolarizing threshold electrotonus and S3 measures are strongly dependent on inward current (Ih) (Tomlinson et al., 2010). Hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels open slowly, then pass a depolarizing Ih after being activated by hyperpolarization. Ih is crucial for regulating the threshold and resting membrane potential both in sensory and motor axons (Shah, 2014). There is strong evidence in the literature for this in human low-threshold motor axons (Trevillion et al., 2010), and that Ih is important in disease pathophysiologies such as diabetes, stroke, porphyria (Kiernan

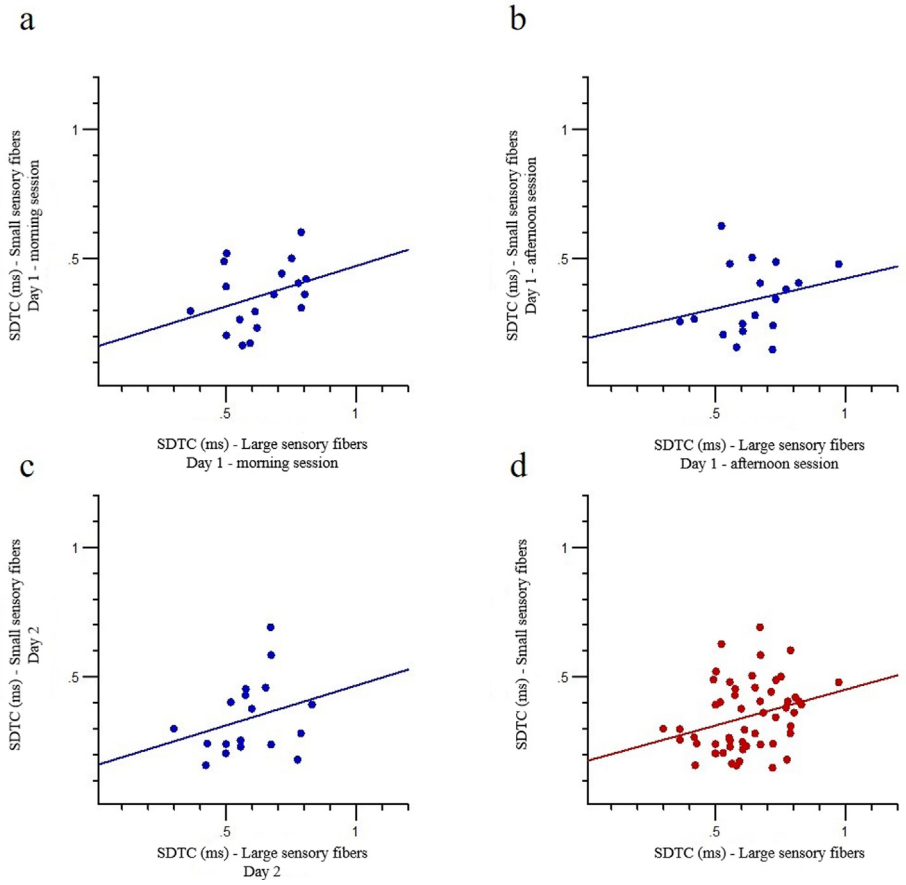

Fig. 3. Correlations for strength-duration-time-constant (SDTC) between large fiber sensory excitability testing and perception threshold tracking. Individual sessions of (a) Day 1, morning session, (b) Day 1, afternoon and (c) Day 2 examinations. (d) All sessions are pooled.

et al., 2020). Although these variables are of great importance, S3 has poor reliability, and should be interpreted cautiously.

One more sensory and motor NET parameter with high variability was refractoriness. Although we paid attention to keeping the temperature between certain limits, this may partly be because of the dependency of this variable to temperature changes (Tankisi et al., 2022). Additionally, rheobase both for sensory and motor NET showed poor reliability. This is a parameter which is not expected to be a reliable measure since it is not only influenced by axonal excitability properties but also stimulus intensity and skin resistance.

For the PTT, the strength-duration relationship parameters and particularly threshold electrotonus showed poor reliability. The strength-duration relationship is mediated by the passive membrane properties of the nodes of Ranvier and the voltage-gated Nav channels (Bostock and Baker, 1988), and SDTC obtained by PTT may be a valuable potential biomarker in pain conditions. Nevertheless, the reliability of SDTC for small fibres was not good as sensory NET. This may be due to the variation in pain perception in general or due to generally low currents applied via the multipin electrodes. There could also be an effect on the results caused by the different relations of overall current to current densities or charge densities for pin and patch electrodes. Despite this larger variability in PTT, we found a significant correlation between small and large sensory fiber SDTC. Despite not measuring the properties of Aδ and C fibers, and based on previous reports showing an association between changes in NET and neuropathic pain or dysesthesias (Misawa et al., 2009; Isose et al., 2010; Heide et al., 2018), this correlation may suggest large sensory fiber SDTC as a potential surrogate biomarker for pain research. Test-retest measurements of small-fiber function in terms of pain thresholds show good test–retest reliability with correlations coefficients of 0.80 for cold pain threshold and 0.88 for heat pain thresholds (Geber et al., 2011). Although we can not make a direct comparison between the reliability of pain thresholds and PTT due to the different statistical methodologies used, pain threshold measurements seem to be more reliable.

Our results indicate that further technical improvements are necessary before we can suggest this parameter as a method for the assessment of small fibers. It is likely that long-duration polarising conditioning stimulus has resulted in activation of larger diameter afferents even at low intensities during the threshold electrotonus recordings. It has been shown before that preferential activation of small nerve fibers through small area electrodes is only possible at low intensities close to the perception threshold (Mouraux and Iannetti, 2018).

This is the first study PTT adapted from conventional NET using automated Qtrac protocols. However, there are studies that used pin electrodes with different configurations than ours, and by using different softwares and programmes. There is a large variation in SDTC among these studies, differing from 0.27 ms to 4.56 ms (Hennings et al., 2017; Hugosdottir et al., 2019; Nielson Hoberg et al., 2019; Poulsen et al., 2020; Tanaka et al., 2020).

Hennings and co-workers compared small and large fiber threshold tracking by activating the small cutaneous nerves electrically using small diameter (0.2 mm) cathodes, and large nerves using ordinary patch electrodes (Hennings et al., 2017). Large fiber SDTC was lower (0.58 ms) than small fiber (1.06 ms) in healthy subjects. While the large fiber SDTC in this study is comparable with our results, we found lower SDTC in small fibers (0.35 ms).

This difference may be due to the different methodologies and programmes. In this study, the reliability of pin and patch electrodes was also examined in a subgroup of healthy subjects at a single 1 ms square pulse SDTC assessment, and the CoV was similar to ours (26.7% for pin electrodes and 6.46% for patch electrodes). The same group found in a later study higher SDTC for pin electrodes (approx. 4.56 ms) (Hugosdottir et al., 2019), and they proposed that one likely reason for this discrepancy between their two studies could be that the shortest stimulation duration was 1 ms in their later study, which might have caused an overestimation of the chronaxie. In a recent study, the group found a SDTC of approx. 0.55 ms (Nielson Hoberg et al., 2019) which is closer to our results. However, further studies with head to head comparisons of the pin electrodes and methodologies are needed to understand the cause of the differences, and improve the methods. In a recent study, the SDTC was found approx. 0.27 ms using slightly a different method by another group (Tanaka et al., 2020).

Our study has some limitations. The number of subjects was low and elderly subjects were not included. Additionally, the study was limited to healthy participants. Furthermore, we do not have any evidence that our multipin stimulation electrodes preferentially activate A-delta fibers. Further studies are needed for this purpose comparing multipin electrode evoked potentials compared with laser evoked potentials similar to other studies compared intra-epidermal electrical stimulation with contact heat stimulation evoked potentials (Lutolf et al., 2022) and electrical stimulation using a concentric planar electrode and laser stimulation using a CO2 laser stimulation (Lefaucheur et al., 2012). Another limitation of our study is that small fibers frequently have habituation, which we unfortunately did not take into account in our study design. This may be another cause of poor reliability.

In conclusion, as biomarkers of peripheral nociceptive signalling, SDTC may be a useful measure but further studies are needed to test this in small fibers. This threshold tracking technique can be applied directly to small sensory fibers via a psychophysical readout, or indirectly via large sensory fibers as surrogates that correlate with small fiber function and have better reliability. This would have high clinical relevance, however, the use of PTT requires technical improvement due to its poor reliability.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Bennedsgaard, K., Ventzel, L., Andersen, N.T., Themistocleous, A.C., Bennett, D.L., Jensen, T.S., Tankisi, H., Finnerup, N.B., 2020a. Oxaliplatin- and docetaxel-induced polyneuropathy: clinical and neurophysiological characteristics. J. Peripher. Nerv. Syst. 25 (4), 377–387.

Bennedsgaard, K., Ventzel, L., Grafe, P., Tigerholm, J., Themistocleous, A.C., Bennett, D.L., Tankisi, H., Finnerup, N.B., 2020b. Cold aggravates abnormal excitability of motor axons in oxaliplatin-treated patients. Muscle Nerve 61 (6), 796–800.

Bostock, H., Baker, M., 1988. Evidence for two types of potassium channel in human motor axons in vivo. Brain Res. 462 (2), 354–358.

Bostock, H., Cikurel, K., Burke, D., 1998. Threshold tracking techniques in the study of human peripheral nerve. Muscle Nerve 21 (2), 137–158.

Bromm, B., Meier, W., 1984. The intracutaneous stimulus: a new pain model for algesimetric studies. Methods Find. Exp. Clin. Pharmacol. 6 (7), 405–410.

De Schoenmacker, I., Berry, C., Blouin, J.S., Rosner, J., Hubli, M., Jutzeler, C.R., Kramer, J.L.K., 2021. An intensity matched comparison of laser- and contact heat evoked potentials. Sci. Rep. 11 (1), 6861.

Farrar, M.A., Lee, M.J., Howells, J., Andrews, P.I., Lin, C.S., 2017. Burning pain: axonal dysfunction in erythromelalgia. Pain 158 (5), 900–911.

Geber, C., Klein, T., Azad, S., Birklein, F., Gierthmuhlen, J., Huge, V., Lauchart, M., Nitzsche, D., Stengel, M., Valet, M., Baron, R., Maier, C., Tolle, T., Treede, R.D., 2011. Test-retest and interobserver reliability of quantitative sensory testing according to the protocol of the German Research Network on Neuropathic Pain (DFNS): a multi-centre study. Pain 152 (3), 548–556.

Gylfadottir, S.S., Itani, M., Kristensen, A.G., Tankisi, H., Jensen, T.S., Sindrup, S.H., Bennett, D., Nyengaard, J.R., Finnerup, N.B., Karlsson, P., 2021. Analysis of Macrophages and Peptidergic Fibers in the Skin of Patients With Painful Diabetic Polyneuropathy. Neurol. Neuroimmunol. Neuroinflamm. 9 (1), e1111.

Heide, R., Bostock, H., Ventzel, L., Grafe, P., Bergmans, J., Fuglsang-Frederiksen, A., Finnerup, N.B., Tankisi, H., 2018. Axonal excitability changes and acute symptoms of oxaliplatin treatment: In vivo evidence for slowed sodium channel inactivation. Clin. Neurophysiol. 129 (3), 694–706.

Hennings, K., Frahm, K.S., Petrini, L., Andersen, O.K., Arendt-Nielsen, L., Morch, C.D., 2017. Membrane properties in small cutaneous nerve fibers in humans. Muscle Nerve 55 (2), 195–201.

Hugosdottir, R., Morch, C.D., Andersen, O.K., Helgason, T., Arendt-Nielsen, L., 2019. Preferential activation of small cutaneous fibers through small pin electrode also depends on the shape of a long duration electrical current. BMC Neurosci. 20 (1), 48.

Isak, B., Tankisi, H., Pugdahl, K., Ventzel, L., Finnerup, N.B., Fuglsang-Frederiksen, A., 2021. Neurophysiologic assessment of small fibre damage in chemotherapy-induced peripheral neuropathy. Clin. Neurophysiol. 132 (8), 1947–1956.

Isose, S., Misawa, S., Sakurai, K., Kanai, K., Shibuya, K., Sekiguchi, Y., Nasu, S., Noto, Y., Fujimaki, Y., Yokote, K., Kuwabara, S., 2010. Mexiletine suppresses nodal persistent sodium currents in sensory axons of patients with neuropathic pain. Clin. Neurophysiol. 121 (5), 719–724.

Kiernan, M.C., Bostock, H., 2000. Effects of membrane polarization and ischaemia on the excitability properties of human motor axons. Brain 123, 2542–2551.

Kiernan, M.C., Bostock, H., Park, S.B., Kaji, R., Krarup, C., Krishnan, A.V., Kuwabara, S., Lin, C.S., Misawa, S., Moldovan, M., Sung, J., Vucic, S., Wainger, B.J., Waxman, S., Burke, D., 2020. Measurement of axonal excitability: Consensus guidelines. Clin. Neurophysiol. 131 (1), 308–323.

Klein, T., Magerl, W., Hopf, H.C., Sandkuhler, J., Treede, R.D., 2004. Perceptual correlates of nociceptive long-term potentiation and long-term depression in humans. J. Neurosci. 24 (4), 964–971.

Krarup, C., Moldovan, M., 2009. Nerve conduction and excitability studies in peripheral nerve disorders. Curr. Opin. Neurol. 22 (5), 460–466.

Kristensen, A.G., Gylfadottir, S., Itani, M., Kuwabara, S., Kroigard, T., Khan, K.S., Finnerup, N.B., Andersen, H., Jensen, T.S., Sindrup, S., Tankisi, H., 2021. Sensory and motor axonal excitability testing in early diabetic neuropathy. Clin. Neurophysiol. 132 (7), 1407–1415.

Kroigard, T., Sodemann, U., Gaist, L.M., Sindrup, S.H., Tankisi, H., 2022. The additional diagnostic value of motor nerve excitability testing in chronic axonal neuropathy. Clin. Neurophysiol. Pract. 7, 27–33.

Lefaucheur, J.P., Ahdab, R., Ayache, S.S., Lefaucheur-Menard, I., Rouie, D., Tebbal, D., Neves, D.O., Ciampi de Andrade, D., 2012. Pain-related evoked potentials: a comparative study between electrical stimulation using a concentric planar electrode and laser stimulation using a CO2 laser. Neurophysiol. Clin. 42 (4), 199–206.

Lutolf, R., Julio, S.U., Schubert, M., Hubli, M., 2022. Intra-epidermal evoked potentials: A promising tool for spinal disorders? Neurophysiol. Clin. 52 (1), 44–57.

Misawa, S., Sakurai, K., Shibuya, K., Isose, S., Kanai, K., Ogino, J., Ishikawa, K., Kuwabara, S., 2009. Neuropathic pain is associated with increased nodal persistent Na(+) currents in human diabetic neuropathy. J. Peripher. Nerv. Syst. 14 (4), 279–284.

Moldovan, M., Alvarez, S., Romer Rosberg, M., Krarup, C., 2013. Axonal voltage-gated ion channels as pharmacological targets for pain. Eur. J. Pharmacol. 708 (1–3), 105–112.

Mouraux, A., Iannetti, G.D., 2018. The search for pain biomarkers in the human brain. Brain 141 (12), 3290–3307.

Nielson Hoberg, T., Frahm, S., Hennings, K., Arendt-Nielsen, L., Dahl Morch, C., 2019. Assessing the modulation of cutaneous sensory fiber excitability using a fast perception threshold tracking technique. Muscle Nerve 60 (4), 367–375.

Nochi, Z., Pia, H., Bloms-Funke, P., Boesl, I., Caspani, O., Chapman, S.C., Fardo, F., Genser, B., Goetz, M., Kostenko, A.V., Leone, C., Li, T., Mouraux, A., Pelz, B., Pogatzki-Zahn, E., Schilder, A., Schnetter, E., Schubart, K., Stouffs, A., Tracey, I., Troconiz, I.F., Truini, A., Van Niel, J., Vela, J.M., Vincent, K., Vollert, J., Wanigasekera, V., Wittayer, M., Tankisi, H., Finnerup, N.B., Phillips, K.G., Treede, R.D., 2022. IMI2-PainCare-BioPain-RCT1: study protocol for a randomized, double-blind, placebo-controlled, crossover, multi-center trial in

healthy subjects to investigate the effects of lacosamide, pregabalin, and tapentadol on biomarkers of pain processing observed by peripheral nerve excitability testing (NET). Trials 23 (1), 163.
Notermans, S.L., 1966. Measurement of the pain threshold determined by electrical stimulation and its clinical application. I. Method and factors possibly influencing the pain threshold. Neurology 16 (11), 1071–1086.
Poulsen, A.H., Tigerholm, J., Meijs, S., Andersen, O.K., Morch, C.D., 2020. Comparison of existing electrode designs for preferential activation of cutaneous nociceptors. J. Neural Eng. 17, (3) 036026.
Shah, M.M., 2014. Cortical HCN channels: function, trafficking and plasticity. J. Physiol. 592 (13), 2711–2719.
Tanaka, S., Gomez-Tames, J., Wasaka, T., Inui, K., Ueno, S., Hirata, A., 2020. Electrical Characterisation of Adelta-Fibres Based on Human in vivo Electrostimulation Threshold. Front. Neurosci. 14, 588056.
Tankisi, H., Bostock, H., Grafe, P., 2022. A test to determine the site of abnormal neuromuscular refractoriness. Clin. Neurophysiol. Pract. 7, 1–6.
Themistocleous, A.C., Kristensen, A.G., Sola, R., Gylfadottir, S.S., Bennedsgaard, K., Itani, M., Kroigard, T., Ventzel, L., Sindrup, S.H., Jensen, T.S., Bostock, H., Serra, J., Finnerup, Tankisi, H., Bennett, D.L.H., 2022. Axonal Excitability Does Not Differ between Painful and Painless Diabetic or Chemotherapy-Induced Distal Symmetrical Polyneuropathy in a Multicenter Observational Study. Ann. Neurol. 91 (4), 506–520.
Tigerholm, J., Hoberg, T.N., Bronnum, D., Vittinghus, M., Frahm, K.S., Morch, C.D., 2020. Small and large cutaneous fibers display different excitability properties to slowly increasing ramp pulses. J. Neurophysiol. 124 (3), 883–894.
Tomlinson, S., Burke, D., Hanna, M., Koltzenburg, M., Bostock, H., 2010. In vivo assessment of HCN channel current (I(h)) in human motor axons. Muscle Nerve 41 (2), 247–256.
Trevillion, L., Howells, J., Bostock, H., Burke, D., 2010. Properties of low-threshold motor axons in the human median nerve. J. Physiol. 588, 2503–2515.
Verdugo, R.J., Matamala, J.M., Inui, K., Kakigi, R., Valls-Sole, J., Hansson, P., Nilsen, K. B., Lombardi, R., Lauria, G., Petropoulos, I.N., Malik, R.A., Treede, R.D., Baumgartner, U., Jara, P.A., Campero, M., 2022. Review of techniques useful for the assessment of sensory small fiber neuropathies: Report from an IFCN expert group. Clin. Neurophysiol. 136, 13–38.