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Running title: Properties of cutaneous nerves

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Running title: Properties of cutaneous nerves

Assessing the modulation of cutaneous sensory fiber excitability using a fast perception threshold tracking technique.

Abstract:

Introduction: Topical application of lidocaine and prilocaine (LP) cream attenuates the functionality of small cutaneous nerve fibers. The aim of this human study was to measure the underlying excitability modulation of small cutaneous nerve fibers using a novel and fast perception threshold tracking (PTT) technique.

Methods: Small sensory fibers were selectively blocked by 120min topical application of LP and confirmed by quantitative sensory testing. Excitability changes of small (activated by a specially designed pin electrode) and large (patch electrode) nerve fibers were assessed as the strength-duration relation and threshold-electrotonus.

Results: The excitability assessed by the strength-duration relation and threshold-electrotonus was significantly modulated for the small ($p<0.05$, Wilcoxon), but not large afferents.

Discussion: This novel PTT technique was able to assess inhibition of membrane properties of small cutaneous fibers suggesting the technique as a diagnostic method for assessing impairment of small fibers as seen in many types of polyneuropathies.

Key words: Quantitative Sensory Testing, perception thresholds, pin electrode, nerve fiber excitability, threshold tracking, cutaneous analgesia, small fiber neuropathy.

1. Introduction

Diagnostic tools for small fiber neuropathy (SFN) are scarce, because activation and assessment of small nerve fibers remain difficult. Small cutaneous fibers have a higher activation threshold than large fibers when electrical stimulation is applied through ordinary large surface patch electrodes [1]. To change this recruitment order of electrical stimulation, alternative pin electrode configurations have been introduced, e.g. as an array of small diameter surface cathodes that generates higher current densities in the epidermis close to the free nerve endings [1-5]. Therefore, the nerve endings of small fibers may be activated at lower intensities than larger fibers as the small nerve fiber endings protrude into the epidermis whereas the large fibers predominantly terminate in dermis [7-8].

The non-invasive threshold tracking technique has been applied to study membrane properties of human peripheral nerves in vivo [9-10]. The threshold tracking technique, however, assesses only large fibers [11]. We have previously developed a novel method for assessing the membrane properties of small fibers by perception threshold tracking (PTT) [12]. With PTT, the strength-duration properties and threshold electrotonus of both small and large fibers were assessed and these membrane properties were shown to be different between the two fiber types [12]. The assessments lasted several hours and the protocol was therefore not clinically feasible. It may be feasible to reduce the number of stimulation pulses while maintaining the information needed to estimate membrane properties of small fibers aiming at diagnosing SFN.

SFN has previously been simulated using topical application of cream containing a

mixture of the local anesthetics lidocaine and prilocaine (LP) [13, 14]. Lidocaine and prilocaine block the voltage gated sodium channels in the cell membrane of the cutaneous nerve fibers [15]. When applied topically, lidocaine and prilocaine diffuse through the skin and are absorbed by the blood vessels in dermis and metabolized. Therefore, topical application of LP cream has a more pronounced effect on small compared to large fibers [16, 17] as it blocks the free epidermal nerve endings before it reaches the large fibers in the dermis [18, 19]. Topical application of LP cream may therefore be considered a surrogate model of SFN as it has a distinct blocking sequence of the cutaneous nerves depending on the application time [17,18].

The aims of this randomized, placebo controlled cross-over study, was to investigate if 1) a reduced and thus faster PTT protocol could be used to evaluate the membrane differences between small and large cutaneous nerve fibers and 2) if the excitability changes in small fibers caused by topical application of LP cream could be detected by this novel PTT protocol.

2. Materials and methods

2.1 Subjects

This study performed on healthy human subjects was approved by the local ethics committee (N-20120046) and conducted according to the declaration of Helsinki. The subjects were given detailed written and verbal information and signed an informed consent form before participating in the study. Exclusion criteria were; a) addiction or prior addiction to cannabis, opioids, or other drugs, b) skin diseases, c) conditions that might lead to peripheral neuropathy, and d) pain relieving medication within the last 48 hours.

2.2 Experimental study design

This was a double-blind, randomized, placebo controlled cross-over methodological study consisting of one experimental session of 4 hours. The subjects were placed in a hospital bed in a comfortable inclined position throughout the session. The forearm rested on a pillow and all stimulations were applied to a 5x5-cm skin area on the volar forearm 5cm distal from the antecubital fossa.

2.3 Perception threshold tracking (PTT)

Electrical stimulation was applied through a cutaneous pin electrode [1, 3, 12] consisting of a circular array of 16 small area cathodes made of blunted stainless steel with a diameter of 0.2mm protruding 1mm from the base of the electrode. A concentric stainless-steel ring with an area of 8.8mm^2 was used as anode (supplementary figure S1A). The cutaneous pin electrode preferentially activates small fibers, i.e. the thinly myelinated Aδ-fibers and to a lesser extend the unmyelinated C-fibers. Large fibers (Aβ-fibers) were activated by ordinary Ag-AgCl surface patch electrodes; a 20 x 15 mm electrode (Neuroline 700; Ambu A/S, Ballerup, Denmark) was used as the cathode and a 5 x 9cm (Pals Neurostimulation Electrode: Axelgaard, CO., Ltd., California) electrode was used as the anode.

PTT was performed with a custom-made program, (LabBench; Aalborg University, Denmark), which controlled an isolated bipolar current stimulator (DS5, Digitimer Ltd, Letchworth Garden City, UK). Perception thresholds were assessed by an adaptive staircase method; stimuli were given with an inter-stimulus interval of 1 second and increased by a step size of 15% until the subject indicated perception by pressing a handheld response button (SMI, Aalborg University, Denmark), then the intensity decreased by 15% until the subject no longer perceived the stimulation (indicated by the subjects releasing the button). In four following sequences, the intensity increased and decreased by 7.5%, 3.5%, 3%, and 3%. The perception threshold was calculated as a

weighted average of all maxima and minima. Each minimum and maximum was weighted by the inverse of the step size, so that minima and maxima assessed with smaller step size weighed more on the estimated threshold.

2.3.1 Stimulation specifications

The PTT protocol used in this study was a reduced version of the protocol used by Hennings et al. [12] therefore; fewer stimuli were applied for the assessment of strength-duration properties and threshold electrotonus protocol. The following stimulations were used: The strength-duration properties were assessed by estimation of perception thresholds to rectangular current pulses of 50μs and 1ms durations. Threshold electrotonus was assessed as the perception threshold reduction by depolarizing conditioning current at 20ms and 80ms and hyperpolarizing conditioning current at 80ms. The conditioning currents had an intensity of 20% of the perception threshold of a 1ms current [12]. The test stimulus was a 1ms rectangular pulse applied at the end of the conditioning pulse.

2.4 Quantitative sensory testing (QST)

The function of vibro-sensitive Aβ-fibers was assessed by determination of the vibration detection threshold (VDT). The function of thermo-receptive Aδ- and C-fibers was

assessed by determination of cold detection threshold (CDT), warm detection threshold (WDT), cold pain threshold (CPT), and heat pain threshold (HPT). The function of mechanoreceptive Aδ-fibers was assessed as the perceived intensity of pinprick stimulations.

2.4.1 Vibration detection threshold (VDT)

VDT was assessed with Somedic Vibrameter® (Somedic AB, Hörby, Sweden) using the method of limits. The amplitude of the 100 Hz vibrations was slowly increased until perception was verbally indicated by the subject. The intensity was then slowly decreased until the vibration was no longer perceived. This procedure was repeated three times and the average the three minima and maxima was used as VDT.

2.4.2 Thermal testing

A 9-cm^2 thermode (ATS, Pathway, Medoc, Ramat Yishai, Israel) was used for thermal stimulation. The baseline temperature was set to 32°C from which the temperature increased or decreased at a rate of 1°C/s. For WDT, the temperature was increased until perception was indicated by the subject by pushing a hand-held stop button. For CDT, the temperature was decreased until perception was indicated by the subject. For CPT, the subject pressed the button when the perception changed from cold into cold pain. For HPT, the subject pressed the button when the perception changed from warmth into heat pain. Each measurement was repeated three times and the averages of these three

measurements were used to determine the CDT, WDT, CPT, and HPT respectively.

2.4.3 Pinprick

Two calibrated pinpricks (weights: 6.4g and 12.8g; Aalborg University, Aalborg, Denmark) were used to stimulate the skin. Each weight was used three times and the subjects rated their perception on a visual analogue scale (VAS, Anchored at 0-no perception, 5-pain threshold, 10-worst imaginable pain).

2.5 Topical application of LP and placebo cream

An even layer of 4 g cream (either a mixture of 2.5% lidocaine and 2.5% prilocaine (LP) cream or placebo cream) was applied to the 5x5-cm skin area on the volar forearm 5cm distal from antecubital fossa under an impermeable plastic occlusive film. After 60 minutes, a cream was applied similarly to the opposite forearm. The plastic film and cream were removed after a total of 120 minutes on each arm. Full effect of LP cream is attained after 120 minutes and remains stable for 60 minutes [17, 18]. Hereafter, PPT and QST assessments were performed within the treated area.

2.6 Blinding and randomization

The LP cream and placebo cream were similar in color, smell, and consistency. The creams were supplied in identical vials and marked in a double blinded manner and were unblinded after data analysis.

The order of the creams (LP or placebo), application side (left or right arm for application of LP cream), order of electrodes (cutaneous pin electrode or patch electrode), and order of PTT and QST assessments were randomized in blocks of four.

2.7 Data analysis

All data analysis and statistical calculations were performed using MATLAB 2015b (MathWorks, Natick, Massachusetts, USA) and SPSS 23 (IBM SPSS Statistics, Armonk, New York, USA). Data are presented as median and interquartile range (IQR). The chronaxie and rheobase were estimated by fitting the perception thresholds of the 50µs and 1ms pulses to Weiss' law [20,21]. This was done by a linear fit between stimulation duration and the charge delivered at perception threshold, i.e. the threshold current multiplied by the stimulation duration (supplementary figure S1B). Following Weiss' law, the chronaxie was defined as the negative intercept with the abscissa and the rheobase as the slope of the regression line. The chronaxie is a measure of nerve fiber excitability and the rheobase is the threshold current of a theoretically infinitely long pulse. The population regression of recordings during placebo is depicted in supplementary figure S1B, individual fits were used for data analysis. Results from

threshold electrotonus are presented as threshold reductions, i.e. the percentage the test pulse threshold was reduced by the conditioning pulse [10].

Wilcoxon signed rank test was used to test for a significant difference of PTT measures (1) between the pin electrode and the patch electrode and (2) between LP and placebo cream.

To assess the ability of the PTT method to distinguish between placebo and LP treated skin, two steps of logistic regression models (LRMs) were established for both electrodes. The chronaxie and rheobase and the three electrotonus threshold reductions were entered as continuous factors. First, a series of univariate LRMs were established for each of the PTT measures. Second, a multifactorial LRM model was established and factor reduction was performed using the forward likelihood-ratio method, to establish an LRM with only independent factors. The accuracy of each LRM was calculated as the percentage of correctly categorized cream types by the LRM.

Krumova et al. [16] reported that the response to topical application of lidocaine differs between subjects. To assess the effect of LP cream on the subjects, the differences between the placebo and LP QST measures were correlated to differences between the placebo and LP PTT measures.

Statistical significance was considered as $p < 0.05$.

3. Results

Thirteen healthy subjects participated in the study; however, one subject was excluded due to failure to complete all recordings. Data collection was completed and analyzed for 12 subjects (5 males, 7 females, age 29.1±8.4 years).

3.1 Different membrane properties of small and large fibers

On the placebo arm, there were significant differences between the cutaneous pin electrode and the patch electrode for chronaxie (547μs; IQR = 470-648μs and 257μs; IQR = 216-344 μs respectively, p = 0.003) and rheobase (0.294mA; IQR = 0.160-0.381mA and 1.044mA; IQR = 0.856-1.162mA respectively, p = 0.002, figure 1). Likewise, there were significant differences in threshold reductions for 80ms depolarizing (p = 0.023,) and 80ms hyperpolarizing (p = 0.002,) threshold electrotonus (figure 2) between the electrodes.

The estimation of the membrane properties lasted approximately 20 min for each fiber type.

3.1.1 The effect of LP cream on membrane properties of afferent fibers

The chronaxie of the small fibers assessed by the pin electrode was significantly reduced after application of LP cream (from 547μs to 338μs, p = 0.019,) and the rheobase was increased (from 0.294mA to 0.588mA, p = 0.002, figure 1). For conditioning currents, there were significant increases for 20ms depolarizing threshold electrotonus (from 6.3% to 25.7%, p = 0.034,) and 80ms depolarizing threshold electrotonus (from -8.3 to

22.3%, p = 0.05,), and a decrease for 80ms hyperpolarizing threshold electrotonus (from 29.4% to –25.5%, p = 0.006, figure 2) for small fibers assessed by the pin electrode. No significant changes of large fiber excitability were observed by PTT assessments of strength-duration relations and threshold electrotonus using the patch electrode (figure 1 and 2).

3.1.2 Logistic regression model

The LRMs showed that while PTT with the patch electrode was not able to categorize LP and placebo better than 50 % correct (chance level), PTT with the cutaneous pin electrode could correctly categorize 87.5% of the trials (Table 1). Furthermore, several of the individual stimulation currents categorized above chance. Factor reduction of the multiple factor LRM showed that the rheobase and the 80ms hyperpolarizing current were independent factors for categorization between placebo and LP treated skin (Table 1).

3.2 Quantitative sensory testing

No significant changes were detected between placebo and LP cream for VDT, CPT, or HPT (Figure 3). Following LP application, the CDT was lower (from 28.4°C to 17.6°C, p = 0.002), WDT was higher (from 33.8°C to 38.4°C, p = 0.002), and the VAS scores to

pinprick stimulation were lower (6.4 g pin from 2.1 cm to 0.3 cm and 12.8 g pin from 2.2 cm to 0.4 cm, both p = 0.002, Figure 3).

3.3 Effect of Lidocaine/Prilocaine (LP) cream

The pinprick ratings and CDT were lower and the WDT was higher on the LP treated arm compared to the placebo arm in all subjects. However, the differences between LP and placebo measures varied between subjects; the pinprick ratings ranged from 0.7 to 3.7 (10-point VAS scale), the CDT from 0.3°C to 24.0°C, and the HDT from 0.1°C to 10.7°C. About half of the subject had a higher VDT, CPT and HPT on the LP arm compared to the placebo arm. The differences between the LP and placebo QST measures were not correlated with the differences between the LP and placebo PTT measures after Bonferroni correction.

4. Discussion

The study showed that the novel and fast PTT protocol could assess the membrane properties of small and large fibers using pin and patch electrodes, respectively. Furthermore, blocking of voltage gated sodium channels using topical application of LP cream could be assessed as a modulation of membrane properties for small fiber but not large fibers. The nerve block of small fibers was confirmed by the findings from the QST assessments.

4.1. Selective assessment of the membrane properties of small and large fibers

Several studies have demonstrated selective activation of small fibers using intraepidermal and small surface electrical stimulation [4, 13, 14, 22] and large fibers using large surface electrical stimulation [1,22]. Previously, Lelic et al. [1] found that when stimulating with this cutaneous pin electrode at up to 10 times the perception threshold, the brain areas processing nociceptive input were activated to a greater extent than when compared to stimulation with a large area surface electrode. These findings were similar to the findings of Inui et al. [4] and Mouraux et al. [22] when using intraepidermal electrical stimulation. However, it is likely that stimulation at high intensities with small surface electrodes and intraepidermal electrical stimulation lead to co-activation of large fibers, thus contaminating the measurements [22]. In the present study, all stimulation intensities were around the perception threshold, therefore co-activation of large fibers was minimized when using the pin electrode.

The observed differences in membrane properties between small and large fibers may relate to the nerve fibers' thickness, degree of myelination and the distribution of ion channels subtypes [23-27]. Studies of small fibers indicate that the tetrodotoxin-resistant sodium channels are preferentially expressed in C-fibers [26,28,29] and possibly unmyelinated intra epidermal ending of Aδ-fibers. This may contribute to the differences in chronaxie between small and large fibers. The present study found a lower chronaxie for small fibers than what was previously observed by Hennings et al. [12] (1060μs ± 690μs). This may be due to different pulse durations used in the two studies. The chronaxie for large fibers in the present study are comparable to what has been found by traditional threshold tracking studies, where the chronaxie for compound sensory potentials is found to be between 318-665μs [9, 21, 30, 31].

Hennings et al. [12] found the rheobase of 0.070 mA ± 0.041 mA for small fibers and 0.43 mA ± 0.10 mA for large fibers which were both lower than the rheobases observed in the present study. This may indicate that the placebo cream used in the present study has affected the conductance of the extracellular medium especially the stratum corneum, hereby changing the impedance.

Threshold electrotonus involves estimating the changes in axonal excitability produced by subthreshold conditioning currents [9]. The threshold electrotonus of large fibers showed a larger threshold reduction for the 20ms depolarizing current compared to the 80ms depolarizing current, similar to previous observations [9, 12, 23]. The depolarizing and unexpectedly, the hyperpolarizing conditioning pulses caused a minor threshold decrease of the small nerve fibers. When assessing the small fibers, some subjects reported a constant sensation and it is possible that they could perceive the

hyperpolarizing conditioning current, thus the current possibly produced direct activation of low-threshold axons and thereby the conditioning current was not subthreshold. This may be due to the hydration of the stratum corneum by the placebo cream [32].

4.2. Blocking of small cutaneous fibers with topical LP cream

Topical application of lidocaine led as expected to significant changes in small-fiber-associated QST thresholds but did not seem to affect large fibers [16, 19]. This was confirmed in the present study, where we found significant reductions in pinprick ratings and CDT (Aδ-fiber function), and a significant increase in WDT (C-fiber function), but no change of VDT (Aβ-fiber function).

Intraepidermal electrical stimulation has also been used to evaluate blocking of small fibers after application of a lidocaine patch, however, large fiber function was not evaluated [14, 15]. In the present study, the QST results support the findings from PTT, where changes were found solely related to the function of the small Aδ- and C-fibers. The logistic regression models confirmed that only small nerve fibers were affected by lidocaine, and that chronaxie, rheobase and 80 ms threshold electrotonus were affected by lidocaine. Factor reduction showed redundance between the measures, rendering the rheobase and 80 ms hyperpolarizing threshold electrotonus independent predictors of lidocaine application.

When PTT was performed on skin treated with LP cream, the thresholds of the small

fibers were similar to the thresholds of the large fibers. This could be due to a complete block of Aδ-fibers and a consequent activation of larger fibers as the stimulation intensities were increased until perception threshold was reached. Yet the results from thermal testing indicate a partial block of small fibers, as the subjects were still able to detect both warm and cold sensations. This was supported by the ratings from pinprick stimulations, which indicate only a partial block of the small mechano-sensitive fibers, in line with the findings of Wehrfritz et al. [19] who found a more pronounced effect of lidocaine on mechano-sensitive fibers compared to thermo-sensitive fibers.

The response to lidocaine differed between subjects. This has been reported previously[16], and indicates that individuals respond differently to lidocaine but may also to some extent relate to moderate reproducibility of QST measures. About half of the subject had an increase of the VDT, CPT and HPT, which could indicate that they were responders to lidocaine. As the changes in VDT, CPT, and HPT were not correlated, the subjects with increased VDT were not the same subject that had increased CPT nor HPT. This indicates that these thresholds were insensitive to LP rather than exhibiting individual differences. As the effect of lidocaine on QST measures did not correlate with the effect of lidocaine on PTT measures, further studies should reveal if PTT measures are redundant to QST measures and if they can predict treatment effects of lidocaine in patients suffering from neuropathic pain.

4.3 Possible clinical implications

In the present study, we chose to apply the LP cream and electrical stimulation at the

volar forearm, because most previous studies using this electrode have applied it here [1, 6, 12]. However, the electrode should be placed as distal as possible if the PTT method is to be used for assessing length-dependent neuropathies. For such application, preferential activation of small sensory fibers by the electrode should be confirmed.

The PTT methodology assessed the membrane properties of small sensory fiber during a 20 min protocol and could therefore potentially be used for diagnosis of SFN. The present study showed that assessment of the rheobase and TE80HYP was sufficient for differentiating between placebo and LP treated skin. Therefore, it would be sufficient only to assess these properties, which would shorten the assessment time even further. However, it is unlikely that the alterations of membrane properties caused by, for example, diabetic neuropathy or chemotherapy induced neuropathy are similar to the alterations caused by topical application of LP cream as described here. Therefore, assessment of small fiber membrane properties must be made for each etiology of SFN, prior to establishing a minimal set of stimulation types that enables diagnosis. Furthermore, for each assessed membrane property, normative values must be established. These normative values will likely need to be controlled for factors such as age.

PTT offers indirect assessments of the membrane properties of small cutaneous nerve fibers. The functionality of small fibers can be assessed by QST, and painful peripheral neuropathies present with different QST profile depending on the etiology [33]. However, early detection of SFN before pain arises may facilitate interventions aimed at the cause of the SFN. SFN may be assessed by intraepidermal nerve fiber density from skin biopsies [34] or corneal nerve fiber densities from confocal microscopy[35]. While these

measures are objective, they do not describe the functionality of the nerve fibers and are poorly related to clinical manifestations such as pain [36-38].

5. Conclusion

This PTT protocol appears to be a robust method for the assessment of membrane properties of both small and large nerve fibers using different surface electrodes. The pin electrode-based PTT protocol might prove useful for diagnosing SFN, as it detected changes in membrane properties of small but not large nerve fibers after selective block of small fibers by topical application of LP cream. This may prove a useful tool in assessments of SFNs such as those due to diabetes and chemotherapy, but will require validation in different SFN entities.

Abbreviations

CDT = Cold Detection Threshold

CPT = Cold Pain Threshold

HPT = Heat Pain Threshold

IQR = Interquartile Range

LP = Liodcaine / Prilocaine

LRM = Logistic Regression Model

PTT = Perception Threshold Tracking

QST = Quantitative Sensory Testing

VDT = Vibration Detection Threshold

WDT = Warm Detection Threshold

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Figures Legends

Figure 1: Median and interquartile range for thresholds to stimulations with rectangular currents, rheobase, and chronaxie for small fiber stimulation with the cutaneous pin electrode (Pin) and large fiber stimulation with the patch electrode (Patch) after application placebo and lidocaine / prilocaine (LP) cream. *$p < 0.05$.

Figure 2: Median and interquartile range for threshold electrotonus expressed as threshold reductions for small fiber stimulation with the cutaneous pin electrode (Pin) and large fiber stimulation with the patch electrode (Patch) after application placebo and lidocaine / prilocaine (LP) cream. *$p < 0.05$.

Figure 3: Median and interquartile range for quantitative sensory testing after application placebo and lidocaine / prilocaine (LP) cream . VAS, Visual analogue scale. *$p < 0.05$.

Table 1. Excitability measures to differentiate between lidocaine/prilocaine and placebo.

	Large fiber excitability					Small fiber excitability				
	Univariate predictive			Multivariate LRM after factor reduction		Univariate predictive			Multivariate LRM after factor reduction	
				Accuracy (%)	54.2				Accuracy (%)	87.5
	Accuracy (%)	Odds ratio	P value	Odds ratio	P value	Accuracy (%)	Odds ratio	P value	Odds ratio	P value
Chronaxie	58.3	1.002 [0.992-1.0012]	0.685	-	-	70.8	0.994 [0.988-1.000]	0.043	-	-
Rheobase	50.0	1.001 [0.998-1.003]	0.486	-	-	87.5	1.008 [1.001-1.015	0.024	1.015 [0.996-1.035]	0.126
TE20DEP	54.2	1.110 [0.983-1.255]	0.092	1.110 [0.983-1.255]	0.092	75.0	1.034 [0.991-1.079]	0.126	-	-
TE80DEP	50.0	1.032 [0.968-1.100]	0.341	-	-	70.8	1.036 [1.002-1.071]	0.036	-	-
TE80HYP	54.2	1.021 [0.978-1.067]	0.345	-	-	83.3	0.943 [0.898-0.989]	0.017	0.925 [0.859-0.996]	0.04

LRM, logistic regression model; TE20DEP, threshold electrotonus 20ms depolarising; TE80DEP, threshold electrotonus 80ms depolarizing; TE80HYP, threshold electrotonus 80ms hyperpolarising.