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Demarcation of secondary hyperalgesia zones: punctate stimulation pressure matters

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Abstract

Background—Secondary hyperalgesia is increased sensitivity in normal tissue near an injury, and it is a measure of central sensitization reflecting injury-related effects on the CNS. Secondary hyperalgesia areas (SHAs), usually assessed by polyamide monofilaments, are important outcomes in studies of analgesic drug effects in humans. However, since the methods applied in demarcating the secondary hyperalgesia zone seem inconsistent across studies, we examined the effect of a standardized approach upon the measurement of SHA following a first degree burn injury (BI).

New Method—The study was a two-observer, test-retest study with the two sessions separated by 6 wks. An observer-blinded design adjusted to examine day-to-day and observer-to-observer variability in SHA was used. In 23 healthy volunteers (12 females/11 males) a BI was induced by a contact thermode (47.0°C, 420 s, 2.5 × 5.0 cm²). The SHA, demarcated by polyamide monofilaments (bending force: 0.2, 69 and 2,569 mN) and a “weighted-pin” stimulator (512 mN), were assessed 45 to 75 min after each BI.

Results—A random effect, linear mixed model demonstrated a logarithmic correlation between elicited skin pressures (mN/mm²) and the SHAs ($P < 0.0001$). No day-to-day or observer-to-observer differences in SHAs were observed. Intraclass correlation coefficients, in the range of 0.51 to 0.84, indicated a moderate to almost perfect reliability between observers.

Comparison with existing methods—No standardized approach in SHA-assessment has hitherto been presented.

Conclusions—This is the first study to demonstrate that demarcation of secondary hyperalgesia zones depends on the developed pressure of the punctate stimulator used.

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Keywords

Burn Injury; Mechanical Hyperalgesia; Monofilaments; Punctate Hyperalgesia; Secondary Hyperalgesia; Sensitization

1. Introduction

The secondary hyperalgesia area (SHA) is a circumscribed zone in normal skin near an injury with enhanced mechanical sensitivity. Secondary hyperalgesia is inducible in the majority of healthy subjects and represents evidence of central sensitization: a stimulus-response enhancing mode that may contribute to the development and maintenance of chronic pain states (Woolf, 2011).

SHA has been a primary outcome measure in a number of experimental studies testing anti-hyperalgesic drugs or investigating physiological pain paradigms (Asghar et al., 2015; Dirks et al., 2002; Letzen et al. 2014; Werner et al., 2004). Induction of secondary hyperalgesia is made by dermal capsaicin administration, repeated electrical stimulation, surgical incision, thermal injury, or by a combination of these methods. Secondary hyperalgesia is a robust and reproducible phenomenon with a duration of several hours (Pedersen et al., 1998a). The borders of the secondary hyperalgesia zone are marked by stimulating from normal skin into the area of secondary hyperalgesia, a transition often described as a normal sensation changing into a burning, pricking or stinging sensation. Mechanical stimulation is either dynamic, by a brush or a cotton bud, or static, by punctate stimuli, using bendable polyamide filaments. Although, allodynic stimuli that do not cause pain in normal skin, but pain in the secondary hyperalgesia zone, are used in SHA-assessments, secondary hyperalgesia is the preferred term (Magerl et al., 1998).

In a recent experimental opioid study we used polyamide monofilaments to demarcate the secondary hyperalgesia zone, induced by a first degree burn injury (Ravn et al., 2013). Interestingly, we were unable to demonstrate any *decrease* in SHAs following morphine-infusions compared to controls, findings contrasting with previous experimental studies employing identical testing paradigms and drug-dosing schemes (Warncke et al., 1997; Warncke et al., 2000). In these studies the authors observed a significant reduction in SHAs of 83% compared to placebo. The only important difference between the studies were the characteristics of monofilaments used in demarcation of the secondary hyperalgesia zones: in our negative study the bending force was 890 mN (91 g) (Ravn et al., 2013) while in the positive studies it was 51 mN (5 g) (Warncke et al., 1997; Warncke et al., 2000). After induction of the burn injury the SHAs in the placebo-group in our negative study were 23 cm² and in the positive study 70 cm². The use of a rigid monofilament with a greater bending force (890 mN) therefore hypothetically could lead to smaller hyperalgesia areas than assessment with a monofilament with lower bending force (51 mN). Therefore the *primary* objective of the present study was during *standardized* conditions, to compare the size of the secondary hyperalgesia zone, demarcated by three different polyamide filaments (0.2, 69 and 2,569 mN) and a “weighted-pin” stimulator (512 mN), following a burn injury. The *secondary* objective was to examine day-to-day and observer-to-observer differences in

measurements of SHAs. In addition, allowing a comparison of our results with the literature, we performed a systematic review of randomized, placebo-controlled studies, examining secondary hyperalgesia induced by a burn injury.

2. Material and methods

2.1 Study

2.1.1 Approval—The study protocol was approved by the Regional Committee on Health Research Ethics (H-1-2013-045) and the Danish Data Protection Agency (30-1097). The study was registered in ClinicalTrials.gov (NCT02286037). The original study-protocol is attached as supplementary data (S1).

2.1.2 Design and Randomization Procedure—The study was a randomized, two-observer, test-retest study with the retest session made in mean (SD) 42 ± 3 days after first study day (Day 1; Fig. 1). The study was single-blinded, i.e., the results were blinded to the volunteers, in regard to the punctate stimulator used and the size of the secondary hyperalgesia zone. The randomization procedure was performed by a registered nurse, not participating in the study, using the randomisation software at www.randomization.org. The volunteers, numbered 1 to 24, were randomly allocated into two groups and each group was then randomly allocated to one of the observers. The observers were allocated to two examination sequences: either early Day 1 and late Day 2, or, late Day 1 and early Day 2 (cf. 2.1.5; Fig. 1). The test-order of the three monofilaments was randomized, while the examinations always ended with the “weighted-pin” stimulator.

2.1.3 Volunteers—Twenty-four volunteers (12 females/12 males) were enrolled in the study. On Day 1 the volunteers were screened according to eligibility (supplementary data S2) and an instant urine drug-test, testing for opioids, was performed.

2.1.4 Observers—Observer 1 (TKR) was a highly proficient investigator with more than four years of experience with quantitative sensory testing, while observer 2 (CE) had only three months of experience.

2.1.5 Study Algorithm—SHAs were assessed 45 to 75 min after induction of the burn injury. Thus, each observer had 15 min to examine SHA: either as an early (45–60 min), or late (60–75 min) examination, determined by randomization between observers on Day 1. On Day 2 the procedure was repeated according to the randomization procedure, however the observer-sequence was changed, balancing time- and sequence-effects. The burn injury was induced on the exact same site as on Day 1.

2.2 Laboratory Environment

The experimental procedures were performed in a quiet, bright room with a temperature range of 23–25°C and a relative humidity (RH) of 31–33%, measured with an indoor thermo-hygrometer with external thermo-probe (THW301, Irox ETG, Bern, Switzerland). The testing sessions were made between 1st of October 2013 and 11th of November 2013,

and were carried out Mondays to Fridays between 07.30 AM and 08.00 PM. The volunteers adopted a comfortable supine position during the assessments.

2.3 Burn Injury

The volunteers were instructed to use a hair trimmer in the assessment area, two days before the study days, in order to avoid interference with the QST-assessments. A rectangular area, $2.5 \times 5.0 \text{ cm}^2$, was delineated with the upper anterior corner 11 cm below the medial meniscus margin of the knee and 6 cm from the anterior margin of the tibia. The first degree burn injury (BI) was induced in the delineated area with a contact thermode (Thermotest, Somedic AB, Hörby, Sweden [active area: $2.5 \times 5.0 \text{ cm}^2$, 47.0°C , 420 s]). The pain intensity during the BI was rated on a visual analogue scale (VAS [0 = no pain, 100 = maximum imaginable pain]) at 0, 30, 60, 120, 180, 240, 300, 360 and 420 seconds after the thermode had reached 47.0°C . The mean pain during the induction of the burn injury was calculated as area-under-the-curve/420 s, using the trapezoidal rule.

2.4 Punctate Stimulators

The different characteristics of the punctate stimulators were chosen in order to get skin pressures just perceivable (just above detection threshold; **PS1**; Table 1), between detection and pain threshold (**PS2**), near pain threshold (**PS3**) and above pain threshold (**PS4**), in hyperalgesic skin (Rasmussen et al., 2015; Ravn et al., 2013). The **PS1–PS3** were polyamide monofilaments (Stoelting Europe, Dublin, Ireland; designated values 2.36, 4.93, 6.65; Table 1) while **PS4** was a “weighted-pin” stimulator (MRC Systems, Heidelberg; 512 mN). The punctate stimulators were calibrated prior to study start using a precision scale (Fx-300i, A&D Weighing, CA, USA; repeatability (SD) 1 mg and linearity $\pm 2 \text{ mg}$ [manufacturer’s information; Table 1]) situated in the laboratory (in regard to ambient temperature and RH cf. 2.2).

2.5 Assessment of Secondary Hyperalgesia Areas

The volunteers were instructed not to move once they were in the correct supine position. A digital camera (Canon Power shot G9, Ota, Japan), secured and locked in a fixed position, perpendicular to the BI-site, was used for repetitive images of individual SHA-demarcations. The border was determined by stimulating along eight symmetric lines converging towards the centre of the burn injury (Ravn et al., 2013). The stimulations started in normal skin outside the area of secondary hyperalgesia and continued inwards toward the BI-area at a rate of 0.5 cm/s delivering a punctate stimulation each 1.5 s. The pressure on the monofilament (**PS1–3**; oriented perpendicularly to the skin) was increased until a slight bending was obtained. Handling of the **PS4** was according to the manufacturer’s recommendations. The volunteers, who had their eyes closed during the assessments, reported the occurrence of a definite change in sensation, to an uncomfortable burning, smarting or stinging sensation. The location was indicated by holding the tip of monofilament pointing to the exact site where hyperalgesia appeared, documenting each corner of the octagon by the digital camera (8 frames per area assessment). The SHAs were calculated (total area with the area of the thermode subtracted) from the octagons using a computer-based vector-algorithm (Canvas 12.0, ACD Systems International, Victoria,

Canada). Assessments of SHAs on Day 1 and Day 2 were made between 45 to 75 min after the BI (Fig. 1).

2.6 Statistics

2.6.1 Sample Size Calculation—An *a priori* sample size calculation was based on the assumptions that a randomized, crossover study design was used, and that data were non-normally distributed, thus a correction factor, ARE (asymptotic relative efficiency), of 0.91 was used (Mumby, 2002). The intra-individual standard deviation for SHAs 60 min after the burn injury was estimated to be 13 cm² (Ravn et al., 2012) and the minimal relevant difference (MIREDF) was set to 16 cm² (estimated from monofilament data with bending forces 50 mN and 890 mN leading to estimated SHAs of 70 cm² and 28 cm², respectively) (Ravn et al., 2013; Warncke et al., 1997; Warncke et al., 2000). Furthermore a significance level (α) of 0.01 and a power (1- β) of 0.90 were chosen. The estimated sample size was 23 intention-to-treat (ITT) subjects, allowing for drop-outs the total number of per-protocol volunteers was set to 25.

2.6.2 Statistical Analyses—The SHA-data were tested for normality with the Kolmogorov-Smirnov test and residual plots. A mixed model with random-effect for subject and fixed-effects for the variables, *stimulus* (natural logarithm **PS**-pressure; Table 2), *study day* (Day 1/Day 2), *examination time* (early/late) and *observer* (observer 1/observer 2) was applied. Since the lightest monofilament **PS1** did not evoke SHAs in any of the assessments (all data were 0), it was concluded that **PS1** was below the detection threshold. Hence, inclusion of **PS1**-data would likely bias the analysis, inferring an incorrect estimation of the effect of PS-pressure would be obtained. Therefore only data from **PS2–4** was used in the mixed regression model. The start model included all two- or three-factor interactions. Non-significant ($P > 0.05$) factors, beginning with interactions, were excluded until all included factors attained significance. Comparisons of paired data, i.e., SHAs comparisons across observers and **PS1–4**, were made with t-tests or Wilcoxon signed-rank test depending on data distribution.

In analyses of *association* between the observers' SHA-assessments, for each punctate stimulator (**PS1–PS4**), the coefficient of determination (R^2) were calculated by univariate correlation analysis (Bland and Altman, 1999). In observer-to-observer *reliability* calculations of intra-class correlation coefficients (ICC (2,1) [two-way random average measures, consistency]) were made for each punctate stimulator (**PS1–PS4**) (Werner et al., 2013a). While there are no agreed upon classifications for levels of reliability for ICCs, for interpretation purposes ICCs were categorized as slight/poor (< 0.2), fair (> 0.2 to 0.4), moderate (> 0.4 to 0.6), substantial (> 0.6 to 0.8) and almost perfect (> 0.8) (Landis and Koch, 1977; Werner et al., 2013b). In analyses of *agreement*, Bland-Altman plots were used (Bland and Altman, 1999). The Bland-Altman plot demonstrates the agreement between test-retest data. The average of the two assessments is plotted against the difference between the two assessments. From this plot the bias (the systematical deviation between the assessments) and the limits of agreement (bias $\pm 1.96 \times$ SD) were calculated for each punctate stimulator (**PS1–PS4**) (Bland and Altman, 1999). As a simple measure of

variability for **PS1–PS4**, the coefficient of variation ($CV = SD/mean$) was calculated. The CVs were compared using one-way ANOVA.

From the null-hypothesis it was inferred that no statistical relationship existed between the SHA-assessments and the applied force or pressure from the punctate stimulators (**PS1–PS4**). The level of significance was set to $p < 0.01$ in the paired data comparison, in order to compensate for type I errors (“false positive” outcomes) due to multiple comparisons.

2.7 Systematic Review

A literature search (TKR) was performed in the database PubMed (search completed December 31, 2014). Only randomized, placebo-controlled, double-blind, experimental studies, including healthy human subjects, examining the effect of analgesics or anti-hyperalgesics on development of secondary hyperalgesia following a BI, were considered. Studies not reporting the force/weight of the polyamide filament used in demarcation of the secondary hyperalgesia zone or the absolute SHA-value during the placebo treatment, were excluded. Non-English studies, abstracts from scientific meetings and material from textbooks were not included. In the search the following terms were used: secondary hyperalgesia AND controlled randomized trials AND (burn OR burn injury OR thermal injury). In case of uncertainty concerning relevance of an article, the subject was discussed between the authors and a final decision was taken by the senior author (MUW). From the 742 records identified (7 articles were retrieved through reference lists and other sources) 38 full-text articles were assessed for eligibility (Fig. 2). The search yielded a total of 22 relevant articles (Brennum et al., 1994; Dirks et al., 2001; Dirks et al., 2002; Eisenach et al., 2002; Eisenach et al., 1998; Holthussen et al., 2000; Lillesø et al., 2000; Mathiesen et al., 2006; Mikkelsen et al., 2001; Mikkelsen et al., 1999; Mikkelsen et al., 2000; Pedersen et al., 1996; Pedersen et al., 1998b; Pedersen et al., 1994; Pedersen et al., 1997; Petersen et al., 2001; Petersen et al., 2003; Ravn et al., 2013; Schulte et al., 2005; Warncke et al., 2000; Werner et al., 2002; Werner et al., 2001), and descriptive data on force/weight of the monofilaments and SHA-values during the placebo treatment, were extracted, tabulated (TKR) and verified independently (TKR, MUW).

3. Results

3.1 Volunteers

Twelve females and 12 males were recruited and 12 females and 11 males completed the study. One male volunteer declined to participate in Day 2 due to acute illness and was therefore excluded from the study. Average age (95% CI) for the volunteers were 23.8 (23.2 to 24.3) yrs and the body mass index (BMI) were 22.6 (21.6 to 23.5) kg/cm².

3.2 Pain during induction of burn injury

The mean (95% CI) pain during induction of the burn injury on Day 1 and Day 2, was 37.1 (25.3 to 41.6) VAS-units and 26.1 (24.0 to 32.3; $P = 0.001$) VAS-units, respectively.

3.3 Secondary Hyperalgesia Areas

While all volunteers responded with measurable SHAs demarcated by **PS2**, **PS3** and **PS4**, no zone was demarcated in any of the volunteers with **PS1** (**Raw research data**). Two volunteers did not develop measurable SHAs with **PS2** on Day 1, and one volunteer did not develop secondary hyperalgesia measurable with **PS2** on Day 2. SHAs were measurable in all volunteers on both Day 1 and Day 2 with **PS3** and with **PS4**. A positive correlation between the applied pressure of **PS2–4** and the size of SHA was evident for both observers (Fig. 3; Table 1). For individual assessments with **PS2** and **PS3** there were no significant differences in SHA across the observers (Fig. 3), while for **PS4** a statistical significant difference between the observers was seen, with larger SHAs for the experienced observer 1 ($P = 0.0005$).

3.4 Mixed effect model

These findings were further corroborated in the more complex mixed effect regression model. No interactions between *PS-stimulus* (natural logarithm punctate stimulation pressure), *study day* (Day 1/Day 2), *examination time* (late/early) and *observer* (observer 1/observer 2) attained significance in the model. Main-effects of **PS-stimulus** ($F = 47.7$, $P < 0.0001$) and examination time ($F = 22.6$, $P < 0.0001$) were seen, while main-effects of study day ($F = 0.75$, $P = 0.39$) and observer ($F = 0.43$, $P = 0.43$) were not significantly different (Table 2).

3.5 Association Analysis

Univariate parametric correlation analyses of SHA-data between observers indicated coefficients of determination (R^2) increasing from 0.24 for **PS2**, 0.45 for **PS3** and to 0.61 for **PS4** (Table 3).

3.6 Reliability Analysis

The observer-to-observer ICCs (95% CI) were for **PS2** 0.51 (−0.17 to 0.79), **PS3** 0.77 (0.45 to 0.90) and **PS4** 0.84 (0.63 to 0.93) indicating levels of moderate to almost perfect reliability, respectively (Table 3).

3.7 Agreement Analysis

The Bland-Altman plot containing SHA-data demarcated by **PS4**, assessed by the two observers, demonstrated a uniform distribution of variability between the observers with a negligible bias (Fig. 4). Limits of agreements between days were for **PS2** $2.5 \pm 42.5 \text{ cm}^2$, **PS3** $-0.7 \pm 54.7 \text{ cm}^2$ and **PS4** $3.2 \pm 66.5 \text{ cm}^2$. Differences calculated in percentage were for **PS2**: $1 \pm 210\%$; **PS3**: $-5 \pm 181\%$ and **PS4**: $5 \pm 185\%$.

3.8 Coefficient of Variation

The CVs were mean (95% CI) for **PS2** 0.76 (0.58 to 0.93), **PS3** 0.57 (0.46 to 0.68) and **PS4** 0.57 (0.42 to 0.71). There were no statistical significant differences in CVs between **PS2–PS4** ($F_{2,66} = 2.34$, $P = 0.10$).

3.9 Violations of Study Protocol

Two violations of the original study protocol were made. *First*, the number of included ITT-subjects was 24, not 25 as stated in the sample size calculation of the protocol, however, the number of per-protocol-subjects was 23 as stated. *Second*, the mixed model with random-effect for subject and fixed-effects for the variables supplementing the *a priori* planned statistical calculations was by our statistician (MAP) considered an appropriate and improved overall statistical model *after* the data collection had been performed.

3.10 Systematic Review

Twenty-two studies assessing secondary hyperalgesia induced by a burn injury, were retrieved ($n = 446$) (Brennum et al., 1994; Dirks et al., 2001; Dirks et al., 2002; Eisenach et al., 2002; Eisenach et al., 1998; Holthusen et al., 2000; Lillesø et al., 2000; Mathiesen et al., 2006; Mikkelsen et al., 2001; Mikkelsen et al., 1999; Mikkelsen et al., 2000; Pedersen et al., 1996; Pedersen et al., 1998; Pedersen et al., 1994; Pedersen et al., 1997; Petersen et al., 2001; Petersen et al., 2003; Ravn et al., 2013; Schulte et al., 2005; Warncke et al., 2000; Werner et al., 2002; Werner et al., 2001). Very few of the studies presented the application procedure of the monofilaments. Figure 5 illustrates the association between the monofilament bending force used in demarcation of the secondary hyperalgesia zone and the corresponding SHAs. Spearman correlation analysis, excluding an outlier (Mathiesen et al., 2006) yielded $\rho = -0.02$ (95% CI: -0.43 to 0.41 ; $P = 0.95$), indicating a complete lack of correlation between the variables.

4. Discussion

The present two-observer, test-retest study is the first to demonstrate a significant correlation between the punctate stimulation pressures used in demarcation of the secondary hyperalgesia zones, and the secondary hyperalgesia areas, following a burn injury in healthy volunteers. The results were reliable across observers and study sessions.

4.1 Clinical Aspects

Since mechanical allodynia is a prominent finding in neuropathic pain states our findings may have clinical implications regarding its demarcation in patients (Treede et al., 1992). Sensory mapping with punctate stimulators are used in various scenarios as diagnostic support or as adjuncts in prediction or monitoring of treatment responses. The polyamide monofilaments are however subject to changes in performance due to a hygroscopic sensitivity, to an impact of the rate of filament application, to the loss of “buckling” force over time and to a critical responsiveness to the angle of application (Andrews, 1993; Bove, 2006; Levin et al., 1978; Werner et al., 2011).

The present study demonstrates that demarcation of secondary hyperalgesia zones with polyamide monofilaments (**PS2** and **PS3**), when performed in a controlled environment and using a standardized testing paradigm, may yield comparable results across observers and, test-days, even when separated by 6 weeks. Although, the statistics on the “weighted-pin” stimulator (**PS4**) seem superior to the polyamide filaments, considering the higher coefficient of determination (R^2) and ICC-value (Table 3), this stimulator could be

considered an alternative in demarcation of static mechanical allodynia. Surprisingly, a significant difference in performance between the experienced and the less experienced observer was demonstrated: the more experienced observer demarcated significantly larger areas with **PS4** compared to **PS3** (Fig. 3). This is somewhat at variance with the results of the mixed effect model showing a significant main-effect of PS-pressure, but no main-effect of observer. Computing the pooled values of SHAs assessed by the observers, a significant difference between **PS3** and **PS4** was still demonstrated ($P = 0.0051$). Since great care was taken to standardize the testing paradigm across the observers, it cannot be excluded that SHA-assessments by the weighted pin instrument, **PS4**, compared to the polyamide monofilament, **PS3**, may require an observer with more extensive experience in sensory testing.

4.2 Physiological Aspects

The present study indicates that the force generated by the punctate stimulators does not correlate well with the SHAs: areas demarcated by **PS4** (512 mN) were significantly larger than for **PS3** (2,569 mN; Table 1, Fig. 3). Our data suggest that for SHA-assessments, punctate pressure (force/area) is a better stimulus parameter than punctate force: this is in agreement with original observations by von Frey from 1896, regarding assessment of pressure and pain thresholds in normal skin, using various ingenious models of animal and human hairs (von Frey, 1896). A number of physiological experimental studies have used non-flexible instruments for punctate stimulation (Magerl et al., 2001; Magerl et al., 1998; Ziegler et al., 1999). The graphical relationship between stimulator area and force is best illustrated by sigmoid shaped curves with the lowest thresholds for detection, sharpness and pain, respectively, obtained with stimulator areas ranging from 0.01 to 0.05 mm² (Greenspan and McGillis, 1991). The sharpness and pain thresholds for a stimulator with an area of 0.05 mm² (comparable to **PS4**) are 49 mN and 390 mN, respectively, and, even much higher for stimulators with areas of 0.2 mm² (**PS2**; 210 mN and 640 mN, respectively) and 1.1 mm² (**PS3**; 1,120 mN and 1,600 mN [pain threshold data extrapolated], respectively).

In a human model of capsaicin-induced secondary hyperalgesia ($n = 9$) the secondary hyperalgesia zones were demarcated by a 20 g (195 mN; 0.34 mm²; 580 kPa) monofilament, an approximation to the **PS2** used in the present study (Huang et al., 2000). Pain ratings were performed from periphery to centre of the capsaicin injection using a servo-controlled electromechanical stimulation instrument equipped with a 0.1 mm wide and 12-mm long, semi-crescent shaped blade (160 to 630 mN). The findings of this study were two-fold. *First* a 37% decrease in pain ratings from the centre to the periphery of the secondary hyperalgesic zone was demonstrated. *Second*, pain ratings dropped precipitously just outside the zone of secondary hyperalgesia, indicating that this sensory disturbance approaches being an “all-or-nothing” phenomenon.

Our findings are conspicuously at odds with this study, since we observed a significant pressure-dependent demarcation of the secondary hyperalgesia zone, contradicting a spatially limited “all-or-none” phenomenon. In the present study the demarcation of the secondary hyperalgesia zone (**PS2–4**) revealed a distinct border from normal to hyperalgesic

skin. In fact, the observation by Huang and co-workers of existence of a gradient of pain perception across the secondary hyperalgesia zone could logically support our findings that demarcation with a high-pressure punctate stimulator would be expected to produce a larger zone than a low-pressure stimulator, not the least, since the statistical model proposed by the Huang et al. (Huang et al., 2000) showed significant differences from normal skin in pain ratings up to 18 cm from the injection centre, clearly exceeding the demarcated secondary hyperalgesia borders. However, a number of differences are apparent between the studies: *first*, the studies used different methods of induction of secondary hyperalgesia, capsaicin injection vs. the BI; *second*, in the study by Huang and co-worker, the stimulation area of the blade was not reported, making inferences about the application pressures quite difficult; *third*, the blade-stimulator, although ingenious in design, does not possess radial symmetry of shape making comparisons of skin sensations with punctate objects quite difficult (or as the authors state “likely provides an alternate means by which to assess “punctate” hyperalgesia”).

Our study raises another important issue, how should the secondary hyperalgesia zone be demarcated? In the capsaicin study cited above, it is not unlikely that different results in the gradient calculations would have been obtained if a punctate stimulator with a high pressure performance, e.g., **PS4** (10,244 kPa), instead of the bendable monofilament (580 kPa, **PS2**; Table 1) had been used. In another elegant study (Magerl et al., 1998), including repetitive (0.2 and 0.6 Hz) punctate stimulation, pain was elicited in nearly all subjects with a blunt² monofilament (area 0.95 mm²; 4,316 kPa) both in normal and in capsaicin-induced hyperalgesic skin. In a recent study (Andersen et al., 2015), with repeated testing sessions, we recently observed that a “weighted-pin” stimulator with a flat tip perpendicular to the longitudinal axis of the pin (verified by microphotograph; 0.049 mm²; 2,612 kPa; 0.2 Hz), only elicited pain in 5 to 10 out of 29 healthy subjects in normal skin, but in 25 to 27 out of 29 subjects in a hyperalgesia zone induced by the BI-model. The non-flexible pin producing the necessary skin pressures seems a more accurate punctate stimulator for demarcation of hyperalgesic zones.

4.3 Statistical Aspects

4.3.1 Association—A statistical significant correlation between the observers was demonstrated in regard to demarcation of secondary hyperalgesia zones with **PS3** and **PS4** (Table 3). This is attributable to the much higher skin pressures vis-à-vis the **PS1** and **PS2**, leading to more well-defined borders of secondary hyperalgesia zone. We would have anticipated a lower data-variability for the high-pressure compared to the low-pressure punctate stimulators, but, although a trend of lower CV's were seen, significance was not attained.

4.3.2 Reliability—The ICC-data demonstrated a high degree of congruency in inter-observer reliability (Table 3). In support of this finding is that no statistical significant differences in SHA-assessments were demonstrated between the observers, for any of the

²The application surface is perpendicular to the longitudinal axis with a slight convex surface.

punctate stimulators (Fig. 3). The highest reliability was in the order **PS4**, **PS3** and **PS2**, inferring a relationship between pressure and reliability, favoring **PS4**.

4.3.3 Agreement—The limits of agreement are very wide (Fig. 4), but the Bland-Altman plot gives an honest and transparent characterization of the data variability. Important for the present study was that no significant observer-related bias for any of the punctate stimulators (**PS2–PS4**) was observed.

4.3.4 Variability between Studies—The systematic review demonstrated a considerable heterogeneity and variability between the studies (Fig. 5): a simple SD in SHAs was 26 cm². If our test-retest data were replicated in new identical studies, we would expect the variability (SD) between these hypothetical studies to correspond to the SEM from the present study, i.e., 1.7 to 3.1 cm² (**PS2–PS4**). This impressive difference quantitates the considerable inter-study heterogeneity.

4.4 Limitations

First, the *a priori* sample size calculation was invalidated since we were not able to reproduce the inverse relationship between the punctate stimulation pressure and SHA, as previously hypothesized. *Second*, the repeated BI-exposure may cause habituation of sensory responses leading to a decrease in heat pain response and a decrease in SHA. Albeit significantly lower pain ratings were seen on Day 2 compared to Day 1, no signs of habituation regarding SHA-assessments, for any of the punctate stimulators, were seen. *Third*, results from the present study only pertains healthy volunteers with normal sensory function, and caution should be taken when extrapolating these results to patients with disease-related sensory deficits.

4.5 Strengths

First, the main strength of the study is the meticulous standardization of the testing paradigm. *Second*, the applied pressures of the punctate stimulators were nearly equidistantly spaced logarithmically (10) (kPa; **PS1** = 1.74; **PS2** = 2.62; **PS3** = 3.40; **PS4** = 4.02). *Third*, in order to avoid observer-bias during the demarcation procedure, each of the corners of the octagon for each punctate stimulator and for each observer, was documented only by digital imaging technique, avoiding skin contact with a marker and leaving no residual sign of the demarcation procedure.

5. Conclusion

The null-hypothesis, inferring that no correlation exists between the performance of the punctate stimulator and the demarcated secondary hyperalgesia zone, was clearly rejected. Interestingly, our systematic review supported the null-hypothesis. However, our study demonstrated a significant correlation between the applied pressures of the punctate stimulators and the demarcated secondary hyperalgesia zones.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BI	first degree burn injury
PS	punctate stimulator
SHA	secondary hyperalgesia

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Highlights

- Secondary hyperalgesia area is a primary outcome in studies of analgesic efficacy
- The literature indicates a lack of standardization in demarcation paradigms
- Standardized demarcation of areas were made with different punctate stimulators
- A highly significant correlation between applied pressure and area was demonstrated
- Standardized assessment methods are recommended in future research

Fig. 1. The study algorithm

The two sessions at Day 1 and Day 2 were identical in testing sequences. The observer-order was randomized between the sessions. Each observer at post-burn times, 45 to 60 min or 60 to 75 min, demarcated secondary hyperalgesia areas by four different punctate stimulators (PS1–PS4). BI = burn injury; Obs. 1 = observer 1.

Fig. 2. CONSORT 2010 Flow Diagram

Fig. 3. Secondary hyperalgesia areas (SHAs)

SHAs (cm²) assessed by punctate stimulator 1 (**PS1**; 0.2 mN), **PS2** (69 mN), **PS3** (2,569 mN) and **PS4** (512 mN) by observer 1 (filled circles, solid line; TKR) and observer 2 (non-filled circles, dashed line; CE), calculated as mean values of Day 1 and Day 2 assessments. Asterisks indicated in the lower part of the graph, relate to observer 1, and the upper part to observer 2. For **PS1–PS3** there were no differences in SHAs between the observers, while for **PS4** a significant difference was seen. *** $P < 0.001$, **** $P < 0.0005$. The whiskers indicate the 95% confidence interval of the median.

Fig. 4. Bland-Altman Plot

The plot illustrates the mean of the secondary hyperalgesia areas (SHAs) between Day 1 and Day 2 (horizontal axis), plotted against the differences in SHAs between Day 1 and Day 2 (vertical axis), for punctate stimulator 4 (**PS4**; 512 mN). The filled circles represent data from Observer 1 (TKR) and the un-filled circles from Observer 2 (CE). The plot illustrates a negligible bias of 3.2 cm^2 and 95% limits of agreement for the SHA-assessments of $3.2 \pm 66.5 cm^2$. Please observe that differences in SHAs between Day 1 and Day 2 include errors due to early vs. late examination times, in addition to observer-related errors and random errors.

Fig. 5. Overview

A systematic overview of randomized, placebo-controlled pharmacological studies using the burn injury model ($n = 446$) (Brennum et al., 1994; Dirks et al., 2001; Dirks et al., 2002; Eisenach et al., 2002; Eisenach et al., 1998; Holthusen et al., 2000; Lilleso et al., 2000; Mathiesen et al., 2006; Mikkelsen et al., 2001; Mikkelsen et al., 1999; Mikkelsen et al., 2000; Pedersen et al., 1996; Pedersen et al., 1998; Pedersen et al., 1994; Pedersen et al., 1997; Petersen et al., 2001; Petersen et al., 2003; Ravn et al., 2013; Schulte et al., 2005; Warncke et al., 2000; Werner et al., 2002; Werner et al., 2001). Circles indicate data from each of the 22 studies: the size of secondary hyperalgesia areas (vertical axis) with corresponding force of the monofilament (horizontal axis) employed in demarcation of the secondary hyperalgesia zone.

Table 1

Characteristics of the punctate stimulators (PS) used in the study: polyamide monofilaments (PS1–PS3) and the “weighted-pin” stimulator (PS4). Designated values and cross-sectional diameters were reported by the manufacturers (PS1–PS3 Stoelting Europe, Dublin, Ireland; PS4 MRC Systems, Heidelberg, Germany). Designated values are log (10) of weight (unit 0.1mg). Weight measurements were made on a precision scale (Ex-300i, A&D Weighing, CA, U.S.A). Force (mN) was calculated as weight (g) × gravity constant (9.81 m/s²; mean ± SD). The actual measured weights (PS1–PS3) deviated with 59% to 89% from the designated values.

	Designated value (g)	Force (mN)	Diameter (mm)	Area (mm ²)	Pressure (kPa)
PS1	2.36 (0.02)	0.2 ± 0.0	0.072	0.004	55.3
PS2	4.93 (8.51)	69.4 ± 0.5	0.460	0.166	417.7
PS3	6.65 (446.68)	2,568.8 ± 10.3	1.143	1.026	2,502.4
PS4	512 -	511.9 ± 0.4	0.250	0.049	10,423.6

Table 2

Secondary hyperalgesia area (SHA) data from the mixed model regression analysis with random-effect for subject and fixed-effects for the variables, *stimulus* (natural logarithm *punctate stimulation* pressure), *study day* (Day 1/Day 2), *examination time* (early [45 to 60 min after burn injury]/late [60 to 75 min after burn injury]) and *observer* (observer 1/observer 2). Estimated SHA (cm²) for **PS2** (418 kPa) assessed at early examination time is calculated as: intercept + ln **PS**-pressure × slope + early $\approx -17.1 + \ln(418) \times 5.0 + 9.1 = 22.2$ cm². Estimated SHA (cm²) for **PS4** (10,424 kPa) assessed at late examination time is calculated as: intercept + ln **PS**-pressure × slope + late $\approx -17.1 + \ln(10,424) \times 5.0 + 0 = 29.2$ cm².

	Fixed-effect variable		Estimate (cm ²)	95% CI (cm ²)
Secondary Hyperalgesia Area	Intercept		-17.1	-29.8 to -4.3
	Stimulus (ln PS -pressure; slope)		5.0	3.6 to 6.5
	Examination Time	Early	9.1	5.3 to 12.9
		Late	0	0

Table 3

A comparison of secondary hyperalgesia areas (SHAs) between observers. Coefficients of determination (R^2 ; 95% CI; P -values) calculated by parametric univariate regression analyses, and intraclass correlation coefficients (ICC; 95% CI) for the punctate stimulators: polyamide monofilaments (**PS1–PS3**) and the “weighted-pin” stimulator (**PS4**). Data were normalized by simple logarithmic transformation. **PS1** did not render any measurable SHA in any assessment. R^2 -values provide a measure of how well the observed SHAs are replicated by the other observer, as the proportion of total variation of the SHAs explained by the model. ICC-values indicated for **PS2** moderate, for **PS3** substantial, and for **PS4** almost perfect reliability.

	R^2 (95% CI; P -value)	ICC (95% CI)
PS1	-	-
PS2	0.24 (0.01 to 0.56; $P = 0.0166$)	0.51 (−0.17 to 0.79)
PS3	0.45 (0.13 to 0.72; $P = 0.0004$)	0.77 (0.45 to 0.90)
PS4	0.61 (0.39 to 0.81; $P < 0.0001$)	0.84 (0.63 to 0.93)