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INTERACTION BETWEEN ULTRAVIOLET B INDUCED CUTANEOUS HYPERALGESIA AND NERVE GROWTH FACTOR INDUCED MUSCLE HYPERALGESIA

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ORIGINAL ARTICLE

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ABSTRACT

Backgrounds and Objectives: Clinical observations indicate that cutaneous hyperalgesia may arise from pain located in deep structures. The objective of this study was to investigate whether combined sensitization of deep and superficial somatic tissues facilitates skin hyperalgesia.

Methods: The interaction between muscle and cutaneous hyperalgesia was investigated in 16 healthy volunteers. Skin sensitization was induced unilaterally on the same randomly selected part of the body by ultraviolet-B (UVB) irradiation above the upper-trapezius and low-back muscles. The next day, muscle hyperalgesia was induced bilaterally in low back muscles by injections of nerve growth factor (NGF). Thus, one day after irradiation there was skin sensitization whereas after two days both skin and muscle sensitizations were present. Cutaneous blood flow, pin-prick thresholds, pressure pain thresholds, temporal summation to repetitive painful pressure stimulation, and stimulus-response functions of graded pressure stimulations and pain intensity were assessed within the irradiated skin area and in the surrounding area before and 1, 2 and 3 days after irradiation.

Results: Comparing baseline with one day after irradiation, UVB and UVB+NGF locations demonstrated: 1) Increased superficial blood flow inside the irradiated area ($P<0.01$); 2) Reduced pin-prick ($P<0.01$) and pressure pain thresholds ($P<0.05$) within the irradiated area and in the surrounding area; 3) Left-shifted pressure stimulus-response function within the irradiated area ($P<0.01$); 4) Facilitated temporal summation inside the irradiated area ($P<0.01$).

Conclusions: Using skin and deep-tissue pain sensitization models simultaneously, no significant synergistic effects were found within the three days investigated suggesting little integration between the two phenomena in this period.

INTRODUCTION

The interaction between sensitization of deep and superficial somatic tissues may escalate hyperalgesic symptoms due to neuronal integration and convergence of central neurons between nociceptive afferent fibers originating from both tissues. Cutaneous hyperalgesia as a result of pain from deeper structures has been reported in osteoarthritis patients (Gwilym et al., 2009; Hayashi et al., 2013) and cutaneous vasomotor responses in the areas of referred muscle pain have been demonstrated experimentally (Lei et al., 2008).

The ultraviolet-B (UVB) inflammatory pain model is a well-established human model inducing stable and prolonged primary hyperalgesia for more than 48 h after irradiation (Gustorff et al., 2004). The inflammation induced by UVB irradiation is characterized by increased cutaneous blood flow, reduced mechanical and thermal pain thresholds (allodynia), and increased responses to suprathreshold stimuli (hyperalgesia) within the area of primary hyperalgesia (Bishop et al., 2009; Bishop et al., 2010). Recently, several studies have demonstrated that UVB may also induce areas of secondary hyperalgesia and allodynia both in animals and humans (Bishop et al., 2010; Davies et al., 2011; Gustorff et al., 2013).

In previous human studies, intramuscular injection of nerve growth factor (NGF) induced a reversible and long-lasting muscle hyperalgesia to pressure stimulation (Andersen et al., 2008; Deising et al., 2012; Svensson et al., 2003; Svensson et al., 2008). NGF is a neurotrophine and an endogenous neuromodulator considered a potential candidate for the explanation of muscle soreness in patients with myalgia (Andersen et al., 2008; Hayashi et al., 2013). NGF is secreted by several structures such as skeletal muscle tissue and works by binding the neuronal trkA receptor (Mendell 1999). A peripheral increase in NGF has been reported as a consequence of inflammation (Amano et al., 1991; Hayashi et al., 2013). During inflammation, NGF is transported back towards the dorsal root ganglion indicating a possible contribution of NGF to central sensitization (Hayashi et al., 2013; Lewin and Mendell 1993). Moreover, NGF induces the nociceptive release of neuropeptides involved in the development of pain (Greco et al., 2008; Woolf et al., 1994).

A recent human study on exercise-induced muscle hyperalgesia and UVB-induced cutaneous hyperalgesia indicates that moderate muscle hyperalgesia may increase the UVB-induced cutaneous blood flow without affecting the UVB-induced cutaneous mechanical hyperalgesia (Lo Vecchio et al., 2015). However, to clarify the combined effects of deep and superficial sensitization, this study investigated whether sensitization of muscle nociceptors by intramuscular injection of NGF affects the UVB-induced cutaneous hyperalgesia. Contrary to the previous study combining exercise-

induced muscle hyperalgesia and UVB sensitization, the applied NGF model avoids potential effects related with to exercise protocol per se (i.e., muscle fatigue and intramuscular blood flow changes).

The aim of the present study was to investigate the combined effects of NGF-induced muscle hyperalgesia below the UVB irradiated skin compared with the effects of UVB irradiation alone by assessment of a few parameters within the UVB irradiated area and in the surrounding area including 1) neurogenic inflammation (blood flow), 2) pin-prick (cutaneous) and pressure (deep somatic) hyperalgesia, and 3) temporal summation to pressure stimulation.

MATERIAL AND METHODS

Subjects

Sixteen healthy subjects of Caucasian descent participated in the study (8 females and 8 males, mean age 25 ± 4 years). Exclusion criteria were any current acute or chronic pain condition, a history of drug abuse, use of analgesics within 1 week, any tattoos or skin diseases in the relevant areas, pregnancy or lactation, participation in more than two back training sessions per week, participation in other clinical trials in the preceding 4 weeks or during the study. Subjects were instructed to avoid UVB exposure during the study period. All subjects received written and oral information about the study and written informed consent was provided. The study was performed in accordance with the Declaration of Helsinki and approved by the regional Ethical Committee (N-20120067).

Experimental protocol

A rectangular area (3x4 cm) above the upper trapezius muscle (approximately 3-5 cm lateral from the spinous process of T4) and another area on the same side at the low back (approximately 3-5 cm lateral from the spinous process of L4) were irradiated with UVB (Fig. S1A). The contralateral mirrored areas were defined as the control locations. The day after the irradiation the subjects received bilateral injections of NGF to induce muscle soreness in the low back (the injection site corresponded to the area defined for UVB irradiation and the control site in the low back) ensuring that the two muscular areas where comparable. The somatosensory sensitivity was assessed at baseline and 24 (before NGF injection), 48 and 72 hours after irradiation. The side for UVB irradiation was chosen randomly and balanced between subjects. The four locations to be assessed were divided into an inner region (3x4 cm, with point 1 in the center) to be UVB irradiated and three outer points (points 2-4). Point 2 was located 2 cm rostral from the edge of the irradiated area and points 3 and 4 were separated by 2 cm (i.e., 4 and 6 cm rostral from the edge of the irradiated skin, Fig S1B). Primary hyperalgesia was measured in point 1 whereas the extent and intensity of the secondary hyperalgesia were measured in points 2-4.

UVB irradiation protocol

A week before the experiment, the minimal erythema dose (MED) for UVB-irradiation was determined for each subject by using a calibrated UVB source (wavelength 290-320nm; Saalmann Multitester, Saalmann, SBC LT 400 Herford, Germany). The MED corresponded to the minimum quantity of UVB light (J/cm2) that produces an erythema with distinct borders 24 h after exposure. Five circular skin areas with a diameter of 1.5 cm at the anterior surface of the right forearm were irradiated with a geometric series of UVB doses ranging from 40 to 100 mJ/cm2. For the experiments, the skin was irradiated using the previously determined MED of each subject multiplied by 3 (Bishop et al., 2009; Gustorff et al., 2004).

NGF-induced muscle soreness

Muscle soreness in the lower back was induced by bilateral injections (one on each side) of recombinant human NGF (0.2 ml, 5µg) prepared by the pharmacy at Aalborg Hospital as described previously (Andersen et al., 2006; Gerber et al., 2011; Hayashi et al., 2013; Svensson et al., 2003). The injections were given into the erector spinae muscle at the level of L4, approximately 3.5 cm lateral to the spinous process. The day after the NGF injections the degree of perceived muscle soreness was evaluated, at the beginning of each session, using a modified 7 points Likert scale anchored with 0: "a complete absence of soreness", 1: "a light soreness in the muscle felt only when touched/a vague ache", 2: "a moderate soreness felt only when touched/a slight persistent ache", 3: "light muscle soreness when lifting objects or carrying objects", 4: "a light muscle soreness, stiffness or weakness when flexing the back", 5: "a moderate muscle soreness, stiffness or weakness when flexing the back", and 6: "a severe muscle soreness, stiffness or weakness that limits my ability to move" (Slater et al., 2003).

Assessment of skin blood flow and mechanical pin-prick thresholds

The skin blood flow was recorded by using laser Doppler imaging technique (Moor LDI2, Devon, UK) whereas the pin-prick pain threshold was assessed using a set of weight-calibrated pin-pricks (Aalborg University, Denmark). Both modalities are reported in the supporting information Methods S1 and have been applied previously (Lo Vecchio et al., 2015).

Assessment of pressure pain sensitivity and temporal summation by pressure algometry

Pressure pain sensitivity, stimulus-response (SR) curve relating the VAS scores and pressure intensity, and temporal summation (TS) were recorded using a computer-controlled pressure algometer (Aalborg University, Denmark), with the modality reported in the supporting information Methods S2. This methodology has also been applied previously (Lo Vecchio et al., 2015).

Statistics

All values are presented as means and standard error of the mean (SEM). Statistical analysis was carried out using SPSS (IBM SPSS ©, V19 2010). The Kolmogorov-Smirnov test for normality was used to test the normality of the data.

In order to maintain the same statistical approach for all parameters, a 3-way repeated measures analysis of variance (ANOVA) was used for each parameter with the following factors: *location* (control, NGF, UVB, and UVB+NGF), *area* (inside (P1) and surrounding area (P2)) and *time* (before irradiation and 24, 48, 72 h after irradiation). In order to test the extension of the area of secondary hyperalgesia for blood flow and for PPT, a 3-way RM-ANOVA was conducted in point 3 and point 4 with the following factors: *location* (control, NGF, UVB, and UVB+NGF), *area* (P3 and P4) and *time* (before irradiation and 24, 48, 72 h after irradiation). The ANOVA was followed by a Bonferroni (Bon) post-hoc test in the case of statistically significant factors or interactions. P-values lower than 0.05 were considered statistically significant.

RESULTS

Effects of UVB

Skin blood flow (BF)

Due to a malfunctioning of the laser Doppler device, blood flow was recorded from 15 of the 16 subjects. Twenty-four hours after the UVB irradiation, the irradiated skin showed a clear erythematic area matching the irradiated area.

Inside (P1): An interaction between location, area and time showed that the BF was significantly increased at 24, 48 and 72 h compared with baseline (ANOVA: $F_{9,6}$= 18.7; P<0.01; Bon: P<0.01; Fig. 2B); the same interaction also showed a difference between 24 h and 72 h (Bon: P<0.01; Fig 2B).

Surrounding area (P2-P4): In P2 an interaction between location, area and time showed that the BF was significantly increased at 24 h compared with baseline (ANOVA: $F_{9,6}$= 18.7; P<0.01; Bon: P<0.05; Fig. 2B). No changes in BF were present in P3 and P4 at any time point (ANOVA: $F_{9,6}$= 12.3; P<0.01; Bon: P>0.16; Fig 2B).

Mechanical pin-prick sensitivity (PP)

Inside (P1) and surrounding area (P2): An interaction between location and time showed that the pin-prick thresholds were significantly decreased at 24, 48 and 72 h compared with baseline (ANOVA: $F_{9,7}$= 8.3; P<0.01; Bon: P<0.01; Fig. 3B). No difference was reported between time points (Bon: P>0.73). A main effect of area indicated that the decrease in mechanical pain threshold inside the irradiated area was higher compared with the surrounding area (ANOVA: $F_{1,15}$= 10.5; P<0.01; Fig. 3B).

Temporal summation

Large probe (2 cm^2 probe):

Inside (P1): An interaction between location, area and time showed that at 24, 48 and 72 h the VAS sum results were significantly increased compared with baseline (ANOVA: $F_{9,7}$= 6.7; P=0.01; Bon: P<0.01; supporting information Fig. S4A). No difference was reported between time points (Bon: P=1).

Surrounding area (P2): An interaction between location, area and time showed that no changes in TS were reported in the surrounding area (ANOVA: $F_{9,7}$= 6.7; P=0.01; Bon: P=1).

Small probe (0.03 cm^2 probe):

Inside (P1): An interaction between area and time showed that 24, 48 and 72 h after irradiation the VAS sum was significantly increased compared with baseline (ANOVA: $F_{3,13}$ = 9.6; P<0.01; Bon: P<0.05; supporting information Fig. S4B). An interaction between location and area also showed that the increase in VAS score was higher than the increase found in the control and NGF locations (ANOVA: $F_{3,13}$ = 14; P<0.01; Bon: P<0.05; supporting information Fig. S4B).

Surrounding area (P2): The interaction between area and times did not show any effect on the VAS sum (ANOVA: $F_{3,13}$ = 9.6; P<0.01; Bon: P>0.45).

Pressure pain sensitivity

Large probe (2 cm^2 probe) and small probe (0.03 cm^2 probe):

Inside (P1): An interaction between location, area and time in the ANOVA showed that the PPTs were significantly reduced at 24, 48 and 72 h compared with baseline (ANOVA: $F_{9,7}$= 7; P<0.01; Bon: P<0.01; supporting information Fig. S5B and Fig. S6B). For the 2cm^2 probe these decreases were larger compared with control and NGF at all time points (Bon: P<0.05).

Surrounding area (P2-P4): The same interaction showed that a decrease in PPTs was also present at 24 h in P2 (ANOVA: $F_{9,7}$= 7; P<0.01; Bon: P<0.05; supporting information Fig. S5B and Fig. S6B). With the 2 cm^2 probe, a time effect showed that at 24 and 48 h a decrease of PPT was present in P3 and P4 compared with baseline (ANOVA: $F_{3,13}$= 5.8, P=0.01; Bon: P<0.05; supporting information Fig. S5B).

Stimulus-response (SR) functions for pressure induced pain

Large probe (2cm^2 probe)

Inside (P1): An interaction between area and time demonstrated that at 24, 48 and 72 h the SR position was significantly left-shifted from baseline (ANOVA: $F_{3,13}$= 5.9, P<0.01; Bon: P<0.01; supporting information Table S1). An interaction between location and area showed that this left-shift was larger compared with the control (ANOVA: $F_{3,13}$= 7.7; P<0.01; Bon: P<0.01). An interaction between location, area and time showed an increased slope at 24, 48 and 72 h (ANOVA: $F_{9,7}$= 5, P<0.05; Bon: P<0.01; supporting information Table S2) with a difference between 24 h and 48 h (Bon: P<0.05).

Surrounding area (P2): An interaction between time and area did not show a change in the SR position (ANOVA: $F_{3,13}$= 5.9; P<0.01; Bon: P>0.73). Further, an interaction between location, area and time showed no change in the SR slope (ANOVA: $F_{9,7}$= 5, P<0.05; Bon: P>0.85; supporting information Table S2)

Small probe (0.03cm^2 probe)

Inside and surrounding area:

An interaction between location and time showed that at 24, 48 and 72 h the SR position was significantly left-shifted from baseline (ANOVA: $F_{9,7}$= 6.6, P<0.05; Bon: P<0.01; supporting information Table S1). An interaction between location and time showed an increased slope at 24, 48 h and 72 h compared with baseline (ANOVA: $F_{9,7}$= 11.8; P<0.01; Bon: P<0.01; supporting information Table S2).

Effects of NGF

NGF-induced soreness

The mean Likert scale scores at 24 and 48 h after NGF injections were 4.4 ± 0.2 and 3.8 ± 0.3, respectively. All subjects had Likert scores above 2 at 24 and 48 h post-injection (with the exception of only two subjects who scored 1 at 48 h after NGF injection). These Likert scores illustrate that at 48 and 72 h after skin irradiation (24 and 48 h post-injection) a condition of deep-tissue soreness was present.

Skin Blood Flow (BF)

Inside (P1): An interaction between location, area and time showed no effect in the cutaneous blood flow at any of the time points (ANOVA: $F_{9,6}$= 18.7; P<0.01; Bon: P>0.058; Fig. 2C).

Surrounding area (P2-P4): No changes in BF were present in P2 (ANOVA: $F_{9,6}$= 18.7; P<0.01; Bon: P>0.05), P3 and P4 at any of the time points (ANOVA: $F_{9,6}$= 12.3; P< 0.01; Bon: P>0.67; Fig 2C).

Mechanical pin-prick sensitivity (PP)

Inside (P1) and surrounding area (P2): An interaction between location and time showed that the pin-prick thresholds were decreased at 48 (ANOVA: $F_{9,7}$= 8.3; P<0.01; Bon P=0.05) and this decrease became significant at 72 h compared with baseline (Bon P<0.05; Fig. 3C). However, these

decreases were lower than the decreases reported in the UVB and UVB+NGF locations (Bon: P<0.05). No differences were reported between time points (Bon: P>0.85).

Temporal summation (TS)

Large probe (2 cm^2 probe):

Inside (P1): An interaction between location, area and time showed that the VAS sum results were significantly increased at 48 and 72 h compared with baseline (ANOVA: $F_{9,7}$= 6.7; P=0.01; Bon: P<0.05; supporting information Fig. S4A). No difference was found between 48 and 72 h (Bon: P=0.58).

Surrounding area (P2): An interaction between location, area and time showed no changes in the surrounding area (ANOVA: $F_{9,7}$= 6.7; P=0.01; Bon: P>0.05; supporting information Fig. S4A).

Small probe (0.03 cm^2 probe):

Inside (P1): An interaction between area and time showed that the VAS sum was significantly increased at 24, 48 and 72 h compared with baseline (ANOVA: $F_{3,13}$ = 9.6; P<0.01; Bon: P<0.05; supporting information Fig. S4B).

Surrounding area (P2): The interaction between area and times showed no effect on the VAS sum (ANOVA: $F_{3,13}$ = 9.6; P<0.01, Bon>0.45).

Pressure pain sensitivity

Large probe (2 cm^2 probe):

Inside (P1): An interaction between location, area and time in the ANOVA showed that the PPTs were reduced at 24 h (ANOVA: $F_{9,7}$= 7; P<0.01; Bon: P<0.05), 48 h (Bon: P=0.05) and 72 h (Bon: P<0.05) compared with baseline. No difference between 24 and 48 and 72 h (one and two days after NGF injections) was found (Bon: P=1; Fig. S5C).

Surrounding area (P2-P4): The same interaction showed that a decrease in PPTs was also present at 24 h in P2 (ANOVA: $F_{9,7}$= 7; P<0.01; Bon: P<0.05; supporting information Fig. S5B). A time effect showed that a decrease of PPT was present in P3 and P4 at 24 and 48 h compared with baseline (ANOVA: $F_{3,13}$= 5.8, P=0.01; supporting information Fig. S5C).

Small probe (0.03 cm^2 probe):

Inside (P1) and surrounding area (P2-P4): An interaction between location, area and time showed no effect in the PPT in any of the points (ANOVA: $F_{9,7}$= 4.4; P<0.05; Bon: P>0.15; supporting information Fig. S6C).

Stimulus-response functions for pressure induced pain

Large probe (2 cm^2 probe):

Inside (P1): An interaction between area and time showed that the SR position was significantly left-shifted at 24, 48 and 72 h compared with baseline (ANOVA: $F_{3,13}$= 5.9; P<0.01; Bon: P<0.01; supporting information Table S1). No difference was reported between 48 and 72 h (one and two days after NGF injections) and at 24 h (Bon: P> 0.20; Table S1). An interaction between location, area and time showed that the SR slope was increased at 24, 48 and 72 h (ANOVA: $F_{9,7}$=5; P<0.05; Bon: P<0.01; supporting information Table S2).

Surrounding area (P2): An interaction between area and time showed no changes in SR position (ANOVA: $F_{3,13}$=5.9; P<0.01; Bon: P>0.73; supporting information Table S1). An interaction between location, area and time showed no change in the slope of the SR function (ANOVA: $F_{9,7}$=5; P<0.05; Bon: P>0.112; supporting information Table S2).

Small probe (0.03 cm^2 probe):

Inside (P1) and surrounding area (P2): An interaction between location and time showed a left-shift of the SR function at 24, 48 and 72 h compared with baseline (ANOVA: $F_{9,7}$=6.6; P<0.05; Bon: P<0.01; supporting information Table S1). An interaction between location and time showed an increased slope at 24, 48 and 72 h compared with baseline (ANOVA: $F_{9,7}$=11.8; P<0.01; Bon: P<0.01; supporting information Table S2).

Combined effect of UVB and NGF

Skin Blood Flow

Inside (P1): At 24, 48 and 72 h the BF was significantly increased compared with baseline (ANOVA: $F_{9,6}$= 18.7; P<0.01; Bon: P<0.01; Fig. 2D). The same interaction showed that at 24 h the decrease in BF was smaller than the decrease reported in the UVB location (Bon: P<0.01) whereas no changes in BF were reported at 48 and 72 h between these two locations (Bon: P>0.31, Fig. 2D).

Surrounding area (P2-P4): No change in cutaneous blood flow was present in P2 (ANOVA: $F_{9,6}$= 18.7; P<0.01; Bon: P>0.239), P3 and P4 at any of the time points (ANOVA: $F_{9,6}$= 12.3; P<0.01; Bon: P> 1; Fig. 2D).

Mechanical pin-prick sensitivity

Inside (P1) and surrounding area (P2): An interaction between location and time showed that the pin-prick thresholds were significantly decreased at 24, 48 and 72 h compared with baseline (ANOVA: $F_{9,7}$= 8.3; P<0.01; Bon: P<0.01; Fig. 3D). No difference was reported between time points (Bon: P>0.20). Moreover, a main effect of area indicated that the decrease in mechanical pain threshold inside the irradiation was higher compared with the surrounding area (ANOVA: $F_{1,15}$= 10.5; P<0.01; Fig. 3D).

Temporal summation

Large probe (2 cm^2 probe):

Inside (P1): An interaction between location, area and time showed that the VAS sum results were significantly increased at 24, 48 and 72 h compared with baseline (ANOVA: $F_{9,7}$= 6.7; P=0.01; P<0.01; supporting information Fig. S4A). No difference was present between time points (Bon: P>0.12). The same interaction showed that at 24 h the increase in TS was higher than the increase reported in the UVB area (Bon: P<0.05) whereas no difference was reported at 48 and 72 h between the two locations (Bon: P=1).

Surrounding area (P2): The same interaction reported in the inside area showed a small but not significant increase of TS at 48 and 72 h compared with baseline (Bon: P=0.054).

Small probe (0.03cm^2)

Inside (P1): An interaction between area and times showed that the VAS sum was significantly increased at 24, 48 and 72 h compared with baseline (ANOVA: $F_{3,13}$ = 9.6; P<0.01; Bon: P<0.05; supporting information Fig. S4B). An interaction between location and area also showed that the increase in VAS score was higher than the increase found in the control and NGF locations (ANOVA: $F_{3,13}$ = 14, P<0.01; Bon: P < 0.05; supporting information Fig. S4B). No difference was found between the UVB+NGF and the UVB area (Bon: P=1).

Surrounding area (P2): The interaction between area and times did not show an effect on the VAS sum (ANOVA: $F_{3,13}$ = 9.6; P<0.01; Bon>0.45).

Pressure pain sensitivity

Large probe (2 cm^2 probe) and small probe (0.03 cm^2 probe):

Inside (P1): An interaction between location, area and time in the ANOVA showed that the PPTs were reduced at 24, 48 and 72 h (ANOVA: $F_{9,7}$= 7; P<0.01; Bon: P<0.05, supporting information Fig. S5D and Fig. S6D) compared with baseline. For the 2 cm^2 probe these decreases were higher compared with control and NGF at all time points (Bon: P<0.05). No difference was present between 24 and 48 and 72 h (one and two days after NGF injections) (Bon: P>0.3). No difference was present between UVB and UVB+NGF locations at any of the time points (P>0.5).

Surrounding area (P2-P4): The same interaction also showed a decrease in PPTs in P2 at 48 and 72 h with the 2 cm^2 probe (Bon: P<0.01, Fig. S5D) but no changes with the 0.03 cm^2 probe (Bon: P>0.13; supporting information Fig. S6D). With the 2 cm^2 probe, a time effect showed that at 24 and 48 h a decrease in PPT was present in P3 and P4 compared with baseline (ANOVA: $F_{3,13}$= 5.8; P=0.01; supporting information Fig. S5D).

Stimulus-response functions for pressure induced pain

Large probe (2 cm^2 probe):

Inside (P1): An interaction between area and time showed a left-shift of the SR function at 24, 48 and 72 h compared with baseline (ANOVA: $F_{3,13}$= 5.9; P< 0.01; Bon: P<0.01; supporting information Table S1). An interaction between location and area also showed that this left-shift was smaller than the left-shift in the UVB and NGF locations (ANOVA: $F_{3,13}$= 7.7; P<0.01; Bon: P<0.01; supporting information Table S1). An interaction between location, area and time showed no change in the slope of the SR function (ANOVA: $F_{9,7}$=5; P<0.05; Bon: P>0.5; supporting information Table S2).

Surrounding area (P2): An interaction between area and time showed no changes in SR position (ANOVA: $F_{3,13}$=5.9; P<0.01; Bon: P>0.73; supporting information Table S1). An interaction between location, area and time showed no change in the slope of the SR function (ANOVA: $F_{9,7}$=5; P<0.05; Bon: P>0.5; supporting information Table S2).

Small probe (0.03 cm^2 probe):

Inside (P1) and surrounding area (P2): An interaction between location and time showed no change in neither the SR position nor the slope (ANOVA: $F_{9,7}$>6.6; P<0.05; Bon:P>0.233; supporting information Table S1and S2).

DISCUSSION

In this study the interaction between deep and superficial sensitization and neurogenic inflammation was assessed utilizing UVB and NGF experimental pain models. Several studies have demonstrated increased skin hyperalgesia in patients with musculoskeletal pain (Helme et al., 1987; Jolliffe et al., 1995; Morris et al., 1998). However, the effect of cutaneous hyperalgesia on deep tissue hyperalgesia when both conditions exist is not fully understood. However, the present study did not show any synergistic effect on the hyperalgesic reactions when the two conditions were applied simultaneously. The two models behaved as previously shown by other authors (Andersen et al., 2008; Davies et al., 2011; Gustorff et al., 2013; Svensson et al., 2008). More information about the two models have been reported in the supporting information Discussion S1.

Skin blood flow responses

Measurement of skin blood flow response was used to assess the degree of neurogenic inflammation. In the present study, the blood flow increased more than 5-fold within the irradiated area 24 h after irradiation and stayed significantly elevated up to 72 h after irradiation. This finding is in line with previous findings showing an 8-fold increase of the blood flow 24 h after irradiation compared with baseline (Lo Vecchio et al., 2014; Lo Vecchio et al., 2015).

Bishop et al. have also demonstrated a significant increase of the vasodilatation after irradiation peaking at 24 h and gradually fading over the next few days (Bishop et al., 2009).

The data collected from the NGF location 24 h after NGF administration did not show any increase in blood flow suggesting that muscle hyperalgesia does not influence cutaneous blood flow.

Mechanical pin-pricks thresholds

Measurement of painful mechanical pin-prick thresholds has been used to detect the degree of cutaneous hyperalgesia. It is well established that the UVB model induces inflammation and peripheral sensitization to mechanical and thermal stimuli (Bishop et al., 2009; Gustorff et al., 2013). In agreement with previous studies, a pronounced cutaneous mechanical hyperalgesia was found within and two cm outside the irradiated area 24 h after UVB irradiation (Bishop et al., 2009; Harrison et al., 2004). This decrease in mechanical pain thresholds remained significantly elevated up to 72 h after irradiation indicating a long-lasting hyperalgesia induced by the UVB model.

The low effect of NGF on the pin-prick thresholds was probably due to the skin sensitization caused by the repetitive stimulations in the area suggesting that the cutaneous mechanical hyperalgesia most likely was developed in response to the UVB model.

Temporal summation of pressure pain

TS of pain mimics the wind-up process in dorsal horn neurons; a phenomenon where the sensitivity of the dorsal horn neurons to C-fiber input can be enhanced by repetitive stimulation at rates above 0.3 Hz (Hayashi et al., 2013). Several studies hypothesized that both UVB and NGF may induce facilitation of central mechanisms (Andersen et al., 2008; Gustorff et al., 2004; Gustorff et al., 2013). Since TS of pain is based on a central mechanism, measurement of TS can be considered a reliable method to test the presence of central sensitization in skin and deep tissues (Arendt-Nielsen et al., 1997). In chronic musculoskeletal pain, TS to repetitive pressure stimulations has been demonstrated to be facilitated compared with healthy controls (Arendt-Nielsen et al., 2010; Staud et al., 2003).

Accordingly, the analysis of the TS applied with the 2 cm^2 probe in the NGF location showed an increased VAS score 48 and 72 h after irradiation (24 and 48 h after NGF injection) showing that NGF induces facilitated TS. The increased VAS score found in the control and NGF locations with the 0.03 cm^2 probe can be explained by the skin sensitization induced by the repetitive testing procedures in these two locations. Moreover, as also stated by other authors, small diameter probes are better designed to stimulate skin nociceptors (Finocchietti et al., 2011; Greenspan and McGillis 1991).

Assessing TS in the UVB irradiated area showed that the VAS scores inside the irradiated area were significantly higher at 24, 48 and 72 h after the irradiation than at baseline, and this result was similar for the two different probes. Since TS of pain is based on a central mechanism, the UVB facilitation of TS indicates involvement of facilitated central mechanisms (Arendt-Nielsen et al., 1994; Nie et al., 2009; Staud et al., 2003). In the UVB+NGF location, the lack of difference in VAS score between 48 h and 72 h after irradiation (one and two days after NGF injections) and 24 h after irradiation indicated no additive effect on the hyperalgesic symptoms when the UVB and NGF models were combined.

Pressure hyperalgesia

The assessments of pressure pain thresholds and SR function to pressure have been used to assess the degree of cutaneous and muscle hyperalgesia. The data collected with both probes showed a significant decrease in PPT within the UVB-irradiated area in line with previous findings suggesting that cutaneous UVB irradiation induced a decrease in mechanical pain thresholds in the skin (Lo Vecchio et al., 2014; Lo Vecchio et al., 2015). A decrease in PPT was also found up to 6 cm from the irradiated area supporting the presence of secondary hyperalgesia induced by the UVB model. A small but significant decrease in PPT was also present in the control and NGF locations and may be due to the skin sensitization induced by the repetitive testing procedures. However, the cutaneous hyperalgesia present in the control sites was significantly lower compared with the magnitude of hyperalgesia in the UVB sites.

The present study showed that the SR curve was left-shifted 24, 48 and 72 h after the UVB exposure with an increased slope inside the irradiated area suggesting that the subjects raised the VAS scale faster than the baseline in response to the skin sensitization induced by UVB (Lo Vecchio et al., 2014). The left-shift results indicate allodynia and hyperalgesia confirming that the UVB irradiation induced primary and secondary hyperalgesia to mechanical pressure stimuli involving sensitization of both peripheral and central mechanisms.

The data collected in the NGF location using the $2cm^2$ probe showed a small effect of NGF on the PPTs. The analysis of the SR function revealed that with both probes the SR curve after NGF was left-shifted with an increased slope 24, 48 and 72 h after irradiation but no difference was found between 24 h after irradiation and 48 and 72 h after irradiation (one and two days after NGF injections, respectively). Interestingly, the SR function in the control location assessed with the $2cm^2$ probe was also affected in a similar manner. According to PPT values and SR function in the NGF location, these results are probably the consequence of the skin sensitization induced by the methods applied.

The present study is the first to expand the NGF model to be used for low back muscles. Interestingly, PPTs were not robustly affected by the NGF injections into the back muscle although the TS of pain was significantly affected. This unique finding together with the previous findings applying NGF in human muscles (e.g., m. tibialis anterior (Andersen et al., 2008; Hayashi et al., 2013; Svensson et al., 2003), m. masseter (Svensson et al., 2008)) can support the idea of different muscle sensitivity profiles in agreement with Weinkauf and collaborators in their most recent study where they mapped the spatial extent of mechanical sensitivity after NGF in two different muscles

finding a difference in NGF hyperalgesia between tibialis anterior and erector spinae muscles in humans (Weinkauf et al., 2014).

In the UVB+NGF location the data collected demonstrated that the decrease in PPT and the left-shift of the SR curve were only due to the skin sensitization effects induced by UVB.

The lack of synergistic effects between cutaneous and deep tissue hyperalgesia should be considered together with the limitations of the study design. For example, it could be speculated that the use of a different muscle pain model such as intramuscular injection of hypertonic saline would have led to a synergistic effect when combined with the UVB cutaneous pain model. It was assumed that the long lasting muscle hyperalgesia induced by NGF may better mimic some clinical pain characteristics than injection of hypertonic saline which does not induce long-lasting soreness (Gerber et al., 2011).

Moreover, in this study the UVB model was always performed 24 h before the NGF injection. A different study design where the NGF injection was performed at least 24 h before the UVB irradiation may have led to a different result and may be recommended for further studies, also in the light of some studies stating that cutaneous hyperalgesia may arise from muscle pain in patients suffering from musculoskeletal pain and osteoarthritis (Helme et al., 1987; Jolliffe et al., 1995; Morris et al., 1998).

CONCLUSION

Within three days no significant synergistic effects were found between cutaneous and nearby muscle hyperalgesia using the UVB and NGF pain models. This suggests that the two manifestations are predominately based on separated mechanisms or are due to individual central mechanisms. These results may give an insight into the mechanism underlying some clinical pain conditions often including both superficial and deep tissue hyperalgesia.

ACKNOWLEDGEMENT

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AUTHOR CONTRIBUTIONS

S.L.V. and T.G.N. contributed substantially to the formulation, design and manuscript drafts. S.L.V. and S.W.C performed the data collection. S.L.V and S.F. performed the statistical analysis. All

authors participated in the interpretation of the data, and reviewed and approved the final version of the manuscript.

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Figure legends

FigS1. Representation of the fours assessments locations (A) on the back and the four points stimulated in each location (B).

Fig2. Mean (± SEM, N = 15) skin blood flow in arbitrary units (AU) before and 24, 48 and 72 h after UVB irradiation inside (P1) and outside (P2) the irradiated area on the four locations. Twenty-four h after irradiation, a significantly increased blood flow was observed in the UVB location and in the UVB+NGF location (P1) compared with baseline values (*, BON: $P < 0.01$). In the UVB location, a small but significant increase was also present outside the irradiated area (P2;*, BON: $P < 0.05$). The cutaneous blood flow inside the irradiation (P1) was still significantly increased for up to 72 h after irradiation (*, BON: $P< 0.01$).

Fig3. Mean (± SEM, N = 16) mechanical pain threshold before and 24, 48 and 72 h after UVB irradiation inside (P1) and outside (P2) the area irradiated on the four locations. Twenty-four h after irradiation, a significant decrease of pin-prick thresholds was observed in the UVB and UVB+NGF locations (*, BON: $P < 0.01$; BD). The mechanical pain thresholds were still significantly decreased for up to 72 h after irradiation ($P< 0.01$). Moreover, in both UVB and UVB+NGF locations, the decrease in mechanical pain threshold inside the irradiation was higher compared with outside area (#, BON: $P < 0.01$; BD).

Fig S4. Mean (± SEM, N = 16) of the sum of temporal summation VAS score at baseline and 24, 48 and 72 h after irradiation assessed with the 2 cm^2 flat probe and 0.03 cm2 probe. In the NGF location, the VAS score calculated with the 2cm^2 probe inside the irradiated area was significantly different from the baseline at 48 and 72 h after irradiation (*, BON: $P < 0.05$), whereas in the UVB and UVB+NGF locations, the VAS score inside the irradiated area 24, 48 and 72 h after irradiation was significantly different from the baseline with both probes (*, BON: $P < 0.05$).

Fig S5. Mean (± SEM, N = 16) pain pressure threshold before and 24, 48 and 72 h after irradiation assessed with the 2 cm^2 flat probe in all the four locations. Twenty-four h after irradiation the PPTs in the UVB and UVB+NGF locations were reduced compared with baseline (*, Bon: $P<0.05$; BD) up to 72h.In the UVB location, a small but significant decrease in PPTs was also present outside the irradiation (P2) 24 h after irradiation (*, Bon: $P<0.05$; B). In the NGF location, 24, 48 and 72 h after irradiation the PPT was decreased compared with baseline (*,$P<0.05$; C). In the UVB+NGF locations, 48 and 72 h after irradiation (one and two days after NGF injection), no difference was reported compared with 24 h (pre-NGF).

Fig S6. Mean (± SEM, N = 16) pressure pain thresholds (PPT) before and 24, 48 and 72 h after irradiation assessed with the 0.03 cm^2 probe in all the four locations. Twenty-four, 48 and 72 h after irradiation the PPT was significantly decreased compared with baseline assessments in the UVB and UVB+NGF locations (*, BON: $P < 0.05$), but no effect on the NGF location was reported.

Figure 2

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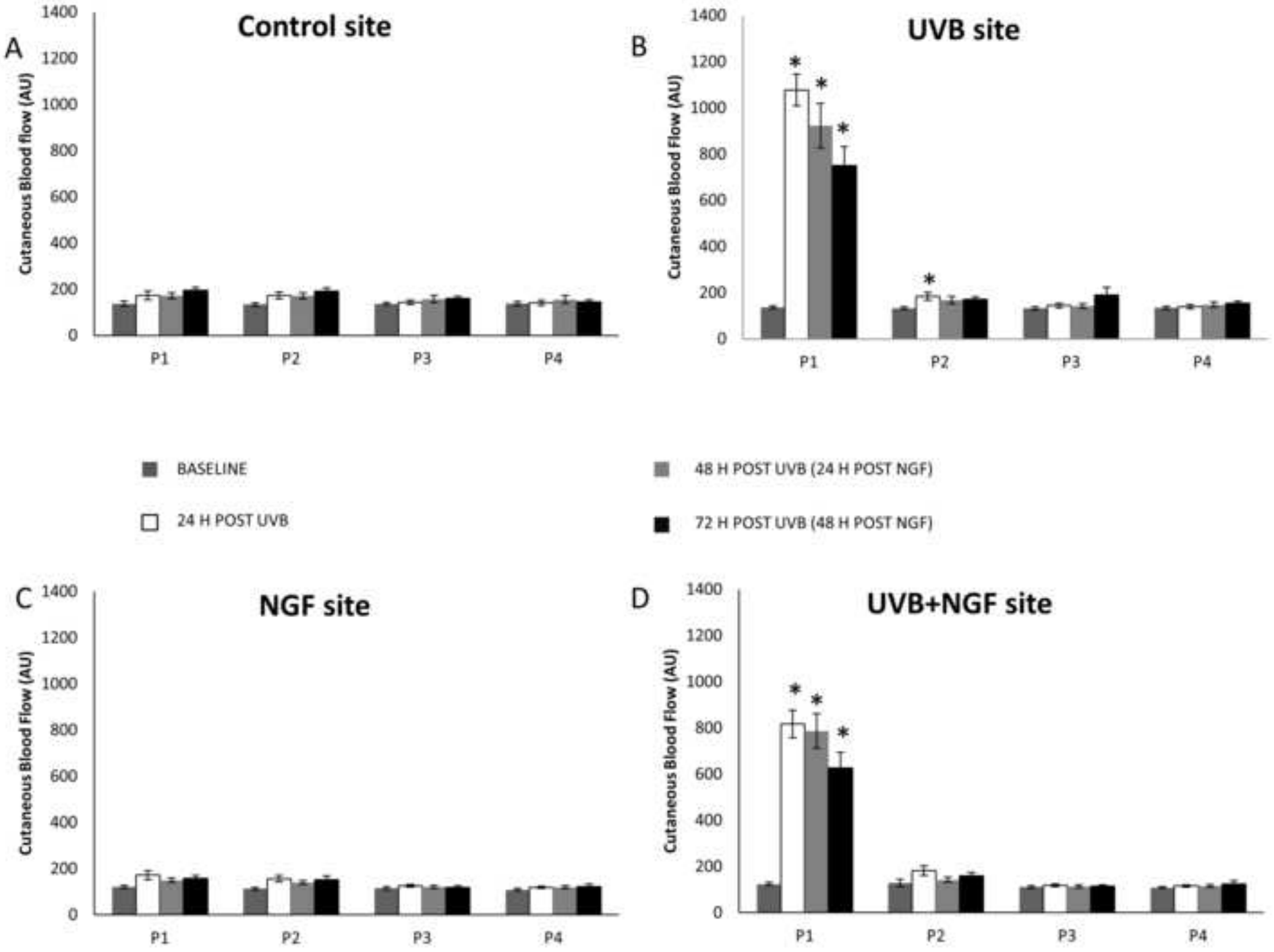

Figure 3

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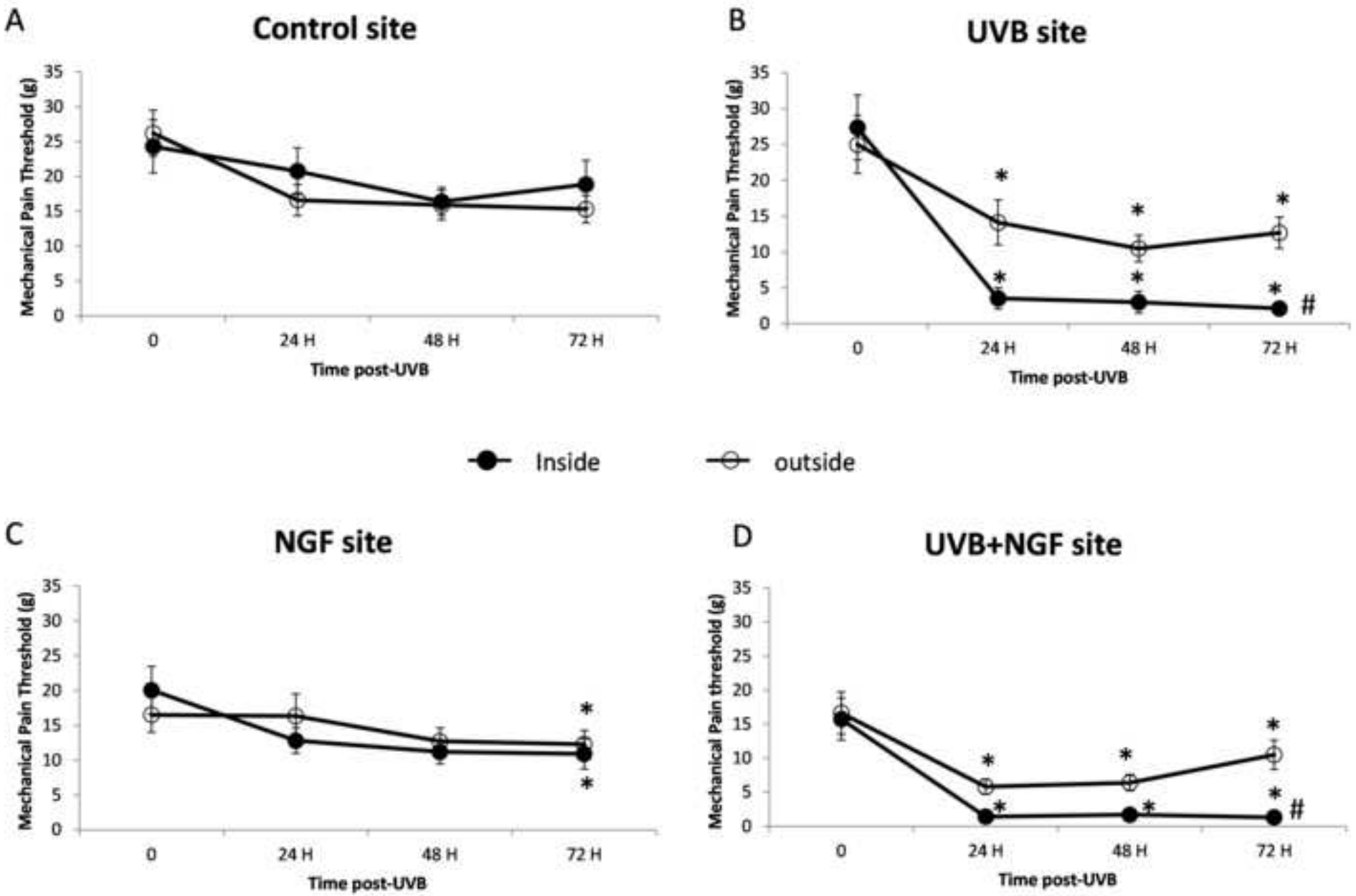

Table S1. Mean (± SEM, N = 16) position of the pressure-VAS stimulus-response curve within (P1) the UVB sites and in adjacent skin (outside, P2) in all 4 locations before, 24, 48 and 72 h after UVB irradiation.

	P1(inside)				P2 (outside)			
	before (kPa)	24 h after (kPa)	48 h after (kPa)	72 h after (kPa)	before (kPa)	24 h after (kPa)	48 h after (kPa)	72 h after (kPa)
Control 2.0 cm^2 probe	275 ± 31	*223 ± 20	*218 ± 22	*222 ± 24	195 ± 13	216 ± 20	198 ± 18	189 ± 17
UVB 2.0 cm^2 probe	229 ± 24	*165 ± 23	*117 ± 15	*129 ± 14	216 ± 30	156 ± 10	185 ± 19	176 ± 17
NGF 2.0 cm^2 probe	276 ± 28	*136 ± 14	*120 ± 14	*131 ± 15	302 ± 40	234 ± 26	204 ± 23	220 ± 24
UVB+NGF 2.0 cm^2 probe	281 ± 24	*249 ± 20	*235 ± 23	*237 ± 24	272 ± 24	243 ± 19	250 ± 22	237 ± 21
Control 0.03 cm^2 probe	5670 ± 355	5274 ± 255	4965 ± 220	4859 ± 239	5400 ± 396	5072 ± 279	5081 ± 252	4809 ± 271
UVB 0.03 cm^2 probe	5727 ± 287	*4306 ± 156	*4211 ± 277	*3972 ± 195	5536 ± 329	*4660 ± 289	* 4537 ± 251	*4684 ± 204
NGF 0.03 cm^2 probe	5576 ± 318	*4273± 157	*4218 ± 167	*4036 ± 155	5784 ± 483	*4818 ± 352	*4743± 267	*4605 ± 290
UVB+NGF 0.03 cm^2 probe	5462 ± 313	5131 ± 185	5070 ± 281	4998 ± 204	5442 ± 331	5054 ± 288	5014 ± 224	4899 ± 155

Significantly decreased compared with the baseline values (*, Bon: $P < 0.05$).

Table S2. Mean (± SEM, N = 16) slope of the pressure-VAS stimulus-response curve within (P1) the UVB sites and in adjacent skin (outside, P2) in all 4 locations before, 24, 48 and 72 h after UVB irradiation.

	P1(inside)				P2 (outside)			
	before (mm/kPa)	24 h after (mm/kPa)	48 h after (mm/kPa)	72 h after (mm/kPa)	before (mm/kPa)	24 h after (mm/kPa)	48 h after (mm/kPa)	72 h after (mm/kPa)
Control 2.0 cm^2 probe	0.304 ± 0.028	*0.377 ± 0.031	*0.421 ± 0.045	0.456 ± 0.064	0.323 ± 0.0028	0.393 ± 0.039	*0.463 ± 0.043	*0.498 ± 0.057
UVB 2.0 cm^2 probe	0.332 ±.0.041	*0.569 ± 0.067	*0.764 ± 0.081	*0.680 ± 0.084	0.376 ± 0.047	0.498 ± 0.049	0.504 ± 0.058	0.520 ± 0.079
NGF 2.0 cm^2 probe	0.308 ± 0.038	*0.668 ± 0.055	*0.744 ± 0.085	*0.688 ± 0.081	0.328 ± 0.038	0.430± 0.059	0.501 ± 0.080	0.471 ± 0.085
UVB+NGF 2.0 cm^2 probe	0.310 ± 0.04	0.341 ± 0.04	0.404 ± 0.06	0.404 ± 0.08	0.313 ± 0.03	0.356 ± 0.04	0.380 ± 0.05	0.408 ± 0.06
Control 0.03 cm^2 probe	0.015 ± 0.001	0.016 ± 0.001	0.017 ± 0.001	0.018 ± 0.001	0.014 ± 0.001	0.016 ± 0.001	0.016 ± 0.001	0.018 ± 0.001
UVB 0.03 cm^2 probe	0.015 ± 0.001	*0.020 ± 0.001	*0.021 ± 0.001	*0.021 ± 0.001	0.014 ± 0.001	*0.018 ± 0.001	*0.018 ±0.001	*0.019 ± 0.001
NGF 0.03 cm^2 probe	0.016 ± 0.001	*0.021 ± 0.001	*0.021 ±0.001	*0.021 ± 0.001	0.015 ± 0.001	*0.018 ± 0.001	*0.018 ± 0.001	*0.019 ± 0.001
UVB+NGF 0.03 cm^2 probe	0.016 ± 0.001	0.017 ± 0.001	0.017 ± 0.001	0.017 ± 0.001	0.016 ± 0.001	0.017 ± 0.001	0.017 ± 0.001	0.018 ± 0.001

Significantly increased slope was observed compared with the baseline values (*, Bon: $P < 0.05$).

Figure S1

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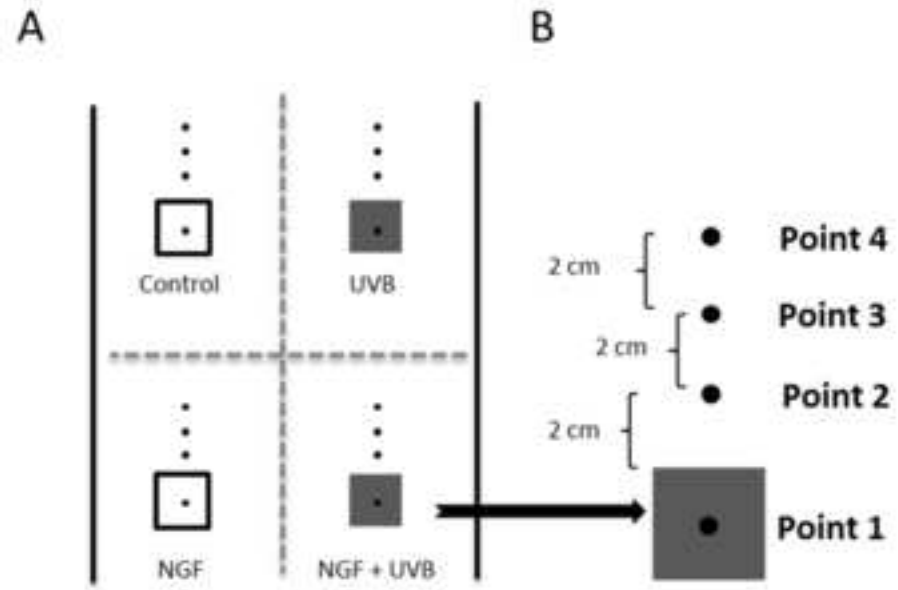

Figure S4

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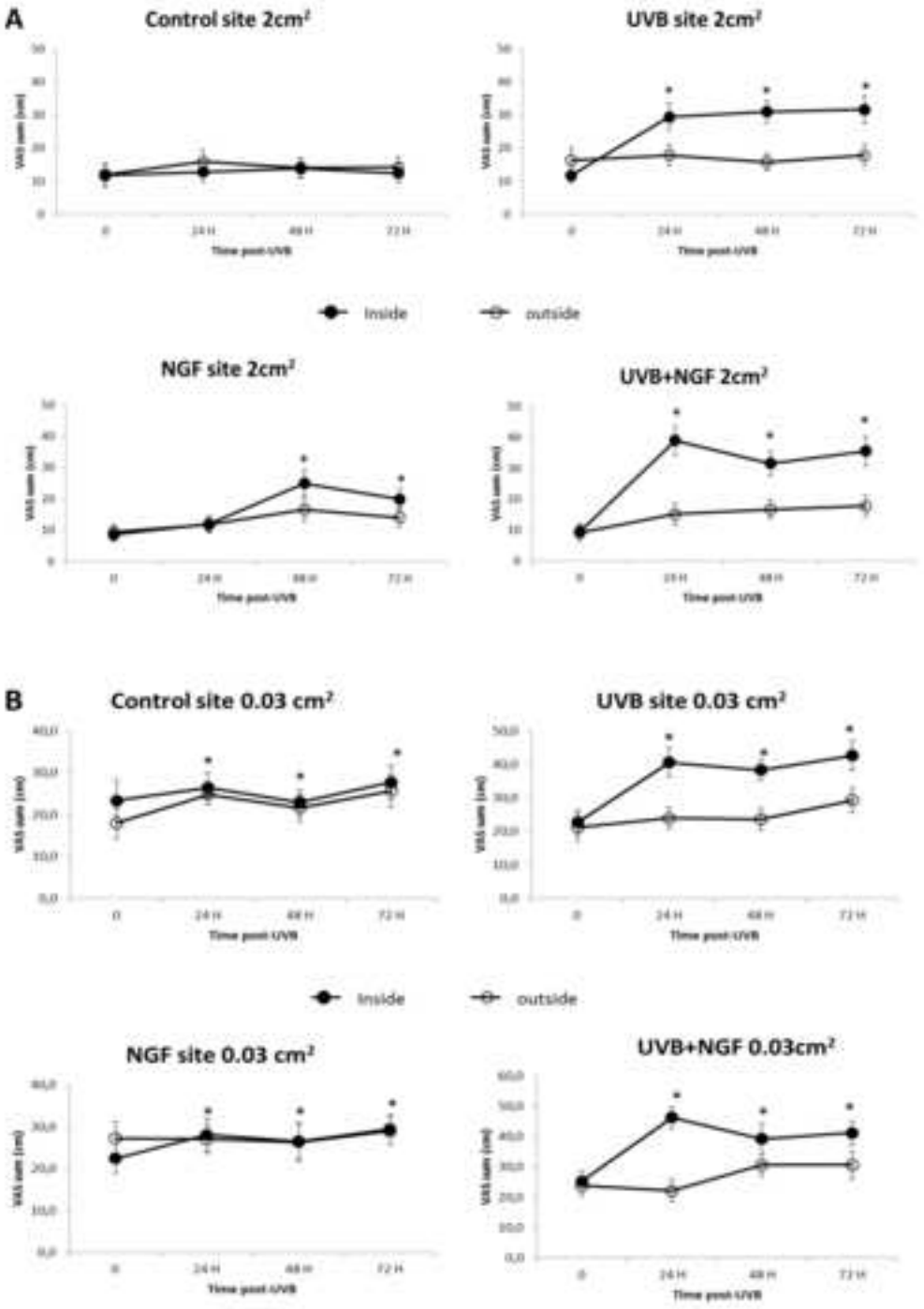

Figure S5

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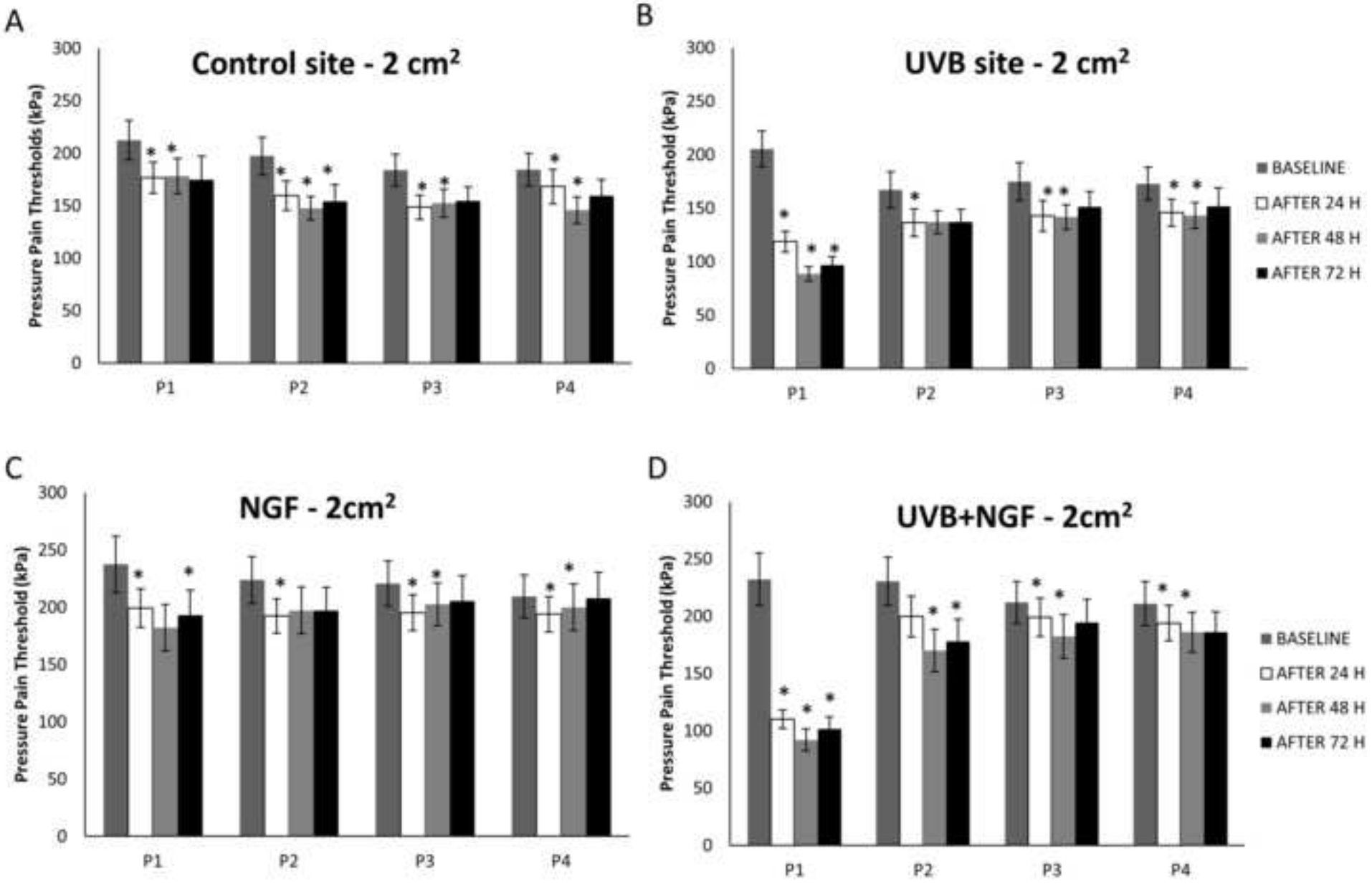

Figure S6

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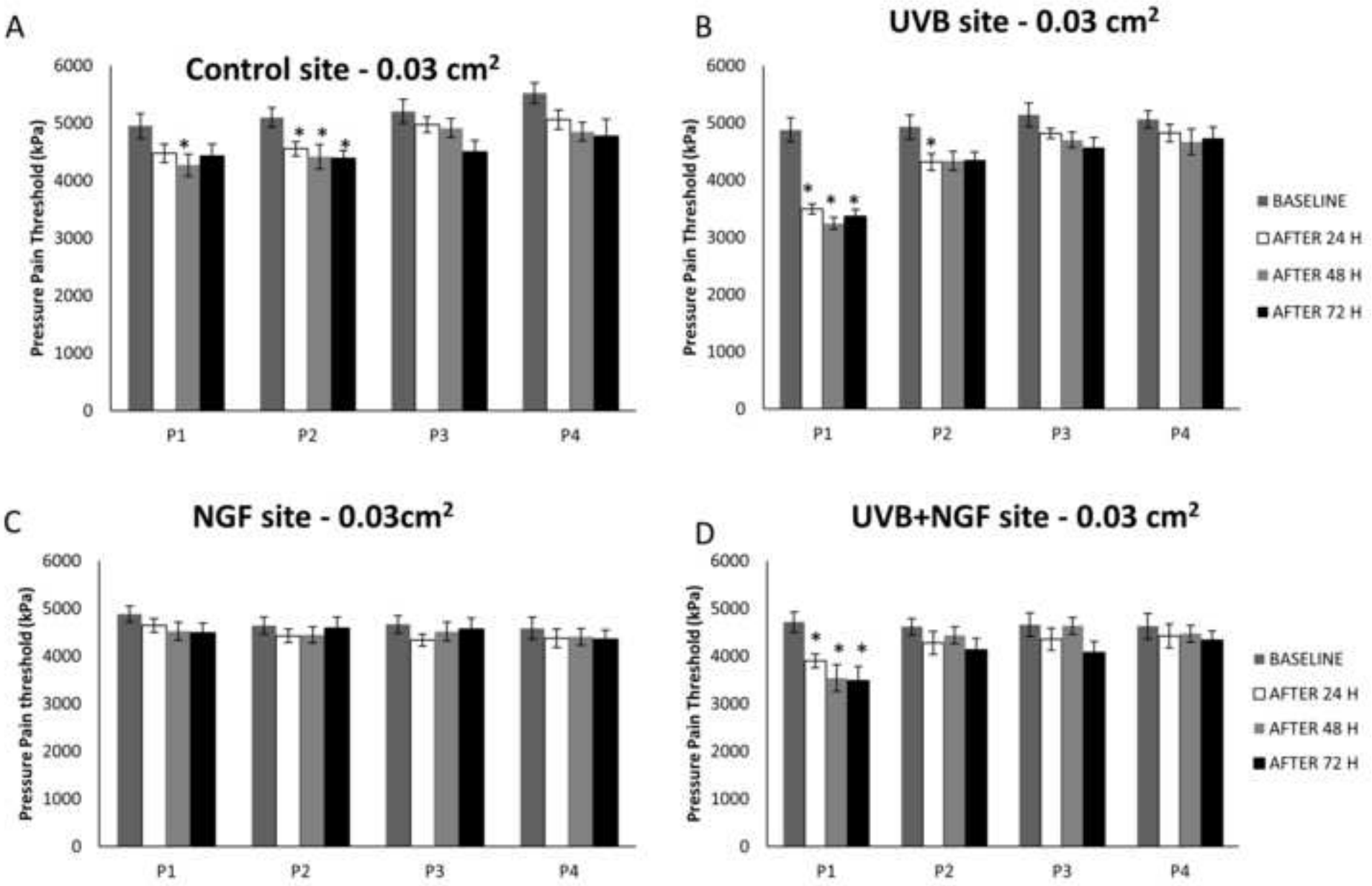

Assessment of skin blood flow

The intensity of the skin erythema was quantified in all locations by measurement of skin blood flow by using laser Doppler imaging technique (Moor LDI2, Devon, UK). The blood flow was assessed at baseline and 24, 48 and 72 h after irradiation. The scan area was 14x5 cm with a 256x256 pixel resolution. The laser head was positioned about 30 cm above the irradiated area and the regions of interest were defined to cover both the irradiated or control area (3x4 cm) and the surrounding skin area by using visible markers. The images were analyzed using dedicated image-processing software (Moor V5.3 Instruments Ltd.) by extracting the mean value of blood flow in 4 different squared areas (2x2 cm) including the 4 assessment points, respectively. The skin blood flow was expressed as arbitrary units (AU).

Weight-calibrated cutaneous pin-prick stimulation

The pin-prick pain threshold was assessed at baseline and 24, 48 and 72 h after irradiation. The measurements were assessed at point 1 and 2 using a set of weight-calibrated pin-pricks (Aalborg University, Denmark). The set consists of 8 metal probes (diameter tip of 0.6 mm) with the following force applications: 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 50.1 and 60.0 g. Starting from the lightest, each of the pin was applied 3 times on the skin (holding the pin for 2 s) until the subject felt a change in sensation from “an innocuous prodding” to a “sharp pricking”. The weight of the pin-prick inducing the "sharp pricking" for at least two of the three stimulations was defined as pin-prick pain threshold.

Assessment of pressure pain sensitivity

The pressure pain sensitivity was recorded using a computer-controlled pressure algometer (Aalborg University, Denmark). The pressure stimulation was applied with 0.3 kg/s perpendicularly to the skin and the subject pressed a button twice: On the first push, the pressure pain threshold (PPT) was recorded and on the second push, the pressure pain tolerance (PPTO) was recorded and the stimulation was stopped. In addition, the subject was asked to rate the pain intensity constantly during the pressure stimulation on an electronic visual analogue scale (VAS) ranging from 0 to 10 cm, where 0 cm indicated ”no pain”, and 10 cm indicated ”maximal pain”. The PPT was defined as the point at which a sensation of strong pressure changed into a sensation of pain. The PPTO was defined as the maximal pressure pain the subject could tolerate. The PPT at all four points was initially measured. Subsequently, the PPTO was recorded at points 1 and 2 and used later to construct the stimulus-response curve. The assessments were performed using two different probes (2.0 cm^2 flat, designed to better assess muscle sensitivity, and a V-shaped probe with a flat contact surface of 0.03 cm^2 at the tip, designed to better assess skin sensitivity). PPT and PPTO were adjusted for the size of the probe by dividing the force threshold by the area of the probe. The points were stimulated in a random order, and all measurements were recorded twice for each point and later the average was used for further analysis.

The stimulus-response (SR) curve relating the VAS scores and pressure intensity was constructed for point 1 and 2, and the pressure equivalent with 5 cm on the VAS was extracted as an estimation of the position of the VAS-pressure curve. This information was used to detect a shift of the stimulus-response curve respect to the baseline recordings. The slope of the VAS-pressure curve was estimated in the VAS range from 0 cm to 10 cm. All pressure algometry parameters were recorded in duplicate and the average was used for further analysis.

Assessment of temporal summation

Temporal summation (TS) of pain to repetitive pressure stimulations was measured at point 1 and 2. The sequential stimulation consists of 10 pressure stimuli (1 s duration, 1 s interstimulus interval) at the pressure pain threshold previously recorded. During the stimulations, the subjects were asked to rate constantly the pain intensity on the VAS. Skin contact between the individual pressure stimuli was ensured by keeping a constant pressure of 0.1 kg; i.e., during the series of sequential stimulation the probe maintained skin contact and was withdrawn after 10 stimulations (Nie et al., 2005). Duplicate measurements for each point were obtained and the mean was used for further

analysis. The VAS score after each stimulus was extracted and the data were normalized by subtracting the VAS score from the first stimulus from the 10 stimuli. The sum of normalized VAS scores (VAS sum) was calculated and used for statistical analysis.

Reference:

Nie H, Arendt-Nielsen L, Andersen H, Graven-Nielsen T. Temporal summation of pain evoked by mechanical stimulation in deep and superficial tissue. The Journal of Pain 2005;6: 348-355.

UVB and NGF models

The results of this study are in line with the findings of previous animals and humans studies (Davies et al., 2011; Gustorff et al., 2013). Present results showed that the UVB model is characterized by increased tissue perfusion and increased mechanical and pressure sensitivity within the area of primary and secondary hyperalgesia.

Our results confirmed that intramuscular injection of NGF induced muscle soreness in humans, in line with the finding of other studies showing that this soreness is characterized by decrease of PPTs after 1 day lasting up to 14 days in some subjects (Andersen et al., 2008; Svensson et al., 2003; Svensson et al., 2008). Moreover, the subjects did not feel any pain during or immediately after injection, but only hours later (Hoheisel et al., 2013). In previous human studies, intramuscular injection of NGF has been used to induce long-lasting hyperalgesia on the tibialis anterior muscle (Andersen et al., 2008; Hayashi et al., 2013), and on the masseter muscle (Svensson et al., 2003; Svensson et al., 2008). This study is one of the first inducing hyperalgesia in the low back muscles in humans. In a previous experiment conducted in rats, Hoheisel and co-workers injected NGF into the multifidus muscle at vertebral level L5 and found that a single injection of NGF caused mechanical allodynia and hyperalgesia, lasting up to 5 days (Hoheisel et al., 2013).

Collectively, it seems that the UVB and NGF models are capable of inducing long lasting hyperalgesic responses in skin and muscle, respectively. However, inter-relationship between the two is not well-investigated yet and the present study, by combining the two models, may help to mimic conditions that have both superficial and deep hyperalgesia involved. More information about the two applied methods can be found on the supporting information discussion S1.

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