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PAIN REFERRAL AREA IS REDUCED BY REMOTE PAIN

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Significance: This study is the first to demonstrate that the size of pain referral can be modulated by engaging endogenous pain inhibitory mechanisms. This method can be used as an approximation to test the sensitivity of central pain mechanisms.

ABSTRACT

Background: Endogenous pain inhibitory mechanisms are known to reduce pain intensity, but whether they influence the size and distribution of pain referral is unclear. This study aimed to determine if referred pain is reduced by applying a remote, conditioning painful stimulus.

Methods: Twenty-four healthy men participated in this randomized, crossover study with a control and conditioning session. Referred pain was induced from the infraspinatus muscle (dominant side) by a painful pressure for 60-s. When applying pressure, the intensity was adjusted to a local pain intensity of 7/10 on a numerical rating scale. In the conditioning session, tonic painful pressure was simultaneously applied to the non-dominant leg during induction of referred pain. The area of referred pain was drawn onto a digital body chart and size extracted for data analysis.

Results: For the total group and in a subgroup with distinct patterns of referred pain (n=15/24), the pain area perceived in the back and front+back, was smaller during the conditioning compared with the control ($P<0.05$). No significant difference was found between sessions in a subgroup only demonstrating local pain (n=9/24).

Conclusions: Engaging the descending noxious inhibitory control reduced the size of pain areas predominately when distinct pain referral was present. Assuming a conditioning effect ~~due~~ of descending inhibitory control acting on dorsal horn neurons, these findings may indicate that mechanisms underlying pain referral can be modulated by endogenous control. The findings may indicate that referred pain may be a useful proxy to evaluate sensitivity of central pain mechanisms as previously suggested.

Significance: The current results indicate a link between endogenous inhibition and pain referral. Descending inhibitory control effects on pain referral support a spinal mechanism involved in pain referral. Future studies should investigate whether the spatial characteristics of referred pain (e.g., size, frequency of affected body regions and distribution away from the primary nociceptive stimulus) can useful to evaluate the efficiency of endogenous pain modulation.

INTRODUCTION

A common phenomenon in musculoskeletal pain is the spread of pain beyond the site of original complaint or injury also known as referred pain (Graven-Nielsen 2006). Referred pain can be experimentally provoked in subjects by applying a noxious stimulus to the somatic structures, such as muscle (Graven-Nielsen 2006), tendon (Drew et al., 2017; Gibson et al., 2006b), ligaments (Palsson et al., 2015; Tsao et al., 2010), joints (Khan et al., 2004; Murakami et al., 2017), and bone (Bastianelli 1939). Any pain subsequently perceived beyond the site of the applied painful stimulus is considered referred pain and can vary in size and distribution. In some cases, healthy individuals only report local pain at the site of the applied stimulus while others report an extensive spread of referred pain (Domenech-Garcia et al., 2016; Doménech-García et al., 2018; Palsson et al., 2020).

There exist a number of protocols for inducing referred pain, such as injection of hypertonic saline (Gibson et al., 2006b), intramuscular electrical stimulation (O'Neill et al., 2009), and suprathreshold pressure stimulation on muscle (Domenech-Garcia et al., 2016; Domenech-Garcia et al., 2018; Gibson et al., 2006b). The size of experimentally-induced referred pain depends on the intensity of the nociceptive drive where the intensity of pain is associated with the size (both total size and distribution away from the primary nociceptive stimulus) of pain referral area in experimental (Domenech-Garcia et al., 2016; Palsson et al., 2015) and clinical conditions (O'Neill et al., 2007; Slater et al., 2005).

Findings from animal studies indicate that peripheral nociceptive drive evoked by the painful stimulus to deep tissue may lead to an unmasking of latent synaptic connections at dorsal horn neurons (Hoheisel et al., 1993). The unmasking of latent synaptic connections is considered a mechanism for enlarging receptive fields of dorsal horn neurons (Hoheisel et al., 1993) and may mediate the spreading of pain in humans (Graven-Nielsen 2006; Mense 1994). Assuming involvement of multi-segmental dorsal horn neurons in the mechanism of referred, it is possible that descending inhibitory control systems may reduce the referred pain distribution without affecting the original noxious stimulus. Descending pain control mechanisms are brainstem-mediated (Bannister and Dickenson 2017; Stroman et al., 2018) and can modulate the sensitivity of postsynaptic wide dynamic range neurons transmitting nociceptive information (Bannister and Dickenson 2017; Ossipov et al., 2014; Villanueva et al., 1984). By applying a distant painful stimulus as conditioning, it is possible to engage a general descending pain inhibition which, in a healthy system, results in a reduction in pain sensitivity. The conditioning pain modulation

effects have been successfully assessed with e.g. the cold pressor (Skovjerg et al. 2017) and mechanical cuff (Graven-Nielsen et al. 2015) as conditioning stimulation.

The purpose of this study was to investigate whether the size of pain referral areas can be modulated by engaging descending inhibitory control mechanisms through a tonic heterotopic pain conditioning stimulus. It was hypothesized that the size of pressure-induced referred pain, would be reduced by a remote painful conditioning stimulus.

METHODS

Participants

Twenty-four male healthy volunteers participated in this study. Inclusion criteria was age between 18 – 40 years, free of pain specific to the shoulder and in general. Exclusion criteria were current or prior serious musculoskeletal injury to and/or surgical interventions, particularly to the shoulder, neck and thoracic spine, current or previous history of substance abuse. Recruitment occurred through advertisements at the university campus and social media platforms. All volunteers received detailed information about the protocol and gave informed consent prior to entering the study. The local Ethics Committee approved this study (N-20150051) which was conducted in accordance with the Helsinki Declaration.

Protocol

This study consisted of two experimental sessions (Control and Conditioning) performed on two consecutive days, separated by at least 24 hours. The sessions were performed in a randomized, balanced cross-over design. Pressure pain sensitivity at the infraspinatus muscle on the dominant side was assessed and participants drew the area(s) of pain onto a digital body chart in response to the tonic painful pressure stimulation. The two sessions were identical with the exception that a secondary and remote painful stimulus was applied to the contralateral (non-dominant) lower leg during the conditioning session. All stimuli were applied by the same member of the research team who has a significant experience with using this stimulation method in research (VDG).

Assessment of pressure pain sensitivity

In the beginning of both sessions, pressure pain thresholds (PPTs) were recorded at the infraspinatus muscle on the dominant side. All assessments and stimuli in both sessions were performed with the subject lying in prone position with the arms down the sides of the body. The assessment site was located by the cut-point of two lines; one coming perpendicular from the mid-part of medial margin of the scapula and the other from the midpoint of the spine of scapulae towards angulus inferior (Domenech-Garcia et al., 2016). The PPT was determined by taking the geometric mean of three PPTs using a handheld pressure algometer (*SBMedic, Sweden*) mounted with a circular probe ($1cm^2$ contact area). The pressure was gradually increased at a constant rate of 30kPa/s until the stimulation became slightly painful. At this point, the subject pressed a button to indicate that the PPT was reached and the pressure was recorded. The PPT values were used to confirm that baseline tissue sensitivity was comparable between sessions.

Experimentally-induced referred pain

To induce experimental referred pain in the shoulder, tonic pressure was applied to the infraspinatus assessment site using the handheld pressure algometer (*SBMedic, Sweden*). As the spreading of pain referral is dependent on the intensity of the nociceptive stimulus (Graven-Nielsen 2006), the stimulus intensity had to be kept constant and compensate for a potential effect of the conditioning **and to ensure that all participants got stimulated at the same relative intensity**. **This way, all participants had the same, relative pain experience.** This was achieved by applying a constant, nociceptive pressure stimulus which caused a local muscle pain rated as 7 out of ten on a numeric rating scale (NRS7). The pressure intensity was gradually increased (30 kPa/s) and when reaching NRS7, the pressure was held steady for 60 seconds. **This stimulation intensity was chosen based on previous findings (Domenech-Garcia et al., 2016) where a stimulation intensity of 5.5-7.5 out of 10 resulted in an extensive pain referral pattern in the majority of subjects.** The subject was instructed to lie still during the whole 60 seconds and relax. The numeric rating scale was positioned on the floor in clear view of the subject during the stimulation. Approximately every 10 seconds, the assessor asked the subject whether the pain intensity had changed from 7/10 **using the question "*How bad is the pain you feel from the pressure on your shoulder?*** If any changes had occurred, the pressure was changed accordingly. The pressure intensity registered at the end of the 60 seconds was extracted

for data analysis. The pressure needed to evoke pain NRS7 was registered in both sessions. This value was expected to increase during the conditioning stimulus and thus reflect increased activity of endogenous pain inhibition (Kennedy et al., 2016). Immediately after releasing the pressure, the subject was asked to draw all the pain area (inside and outside the stimulation site on the front and back side) on a digital body chart *(Navigate Pain, Aalborg University, Denmark)*(Boudreau et al., 2014).

Based on the pain drawing data, the total size of the referred pain area (expressed as pixels) was extracted for the total group. As referred pain is defined as pain occurring outside the stimulation site (Arendt-Nielsen and Svensson 2001), only pain external to the boundaries of the medial and lateral scapular borders, angulus inferior below, and the spine of the scapula above was considered to be referred pain. This process was done by visual inspection of the body charts in the post-processing phase. For the referred pain group, the area size and spread of the referred pain area was extracted.

The distribution of referred pain on the digital body chart was post-processed to calculate the maximum length or radiating spread of pain from the ipsilateral earlobe to the most distally located pixel on the body chart. The purpose of calculating the radiating spread was to determine any change, proximal or distal, in pain distribution when inducing a remote, painful stimulus. The earlobe was chosen since it is an anatomical landmark clearly identifiable from both the front and back view on the body chart.

Remote painful stimulation

A cuff algometer (*NociTech, Aalborg, Denmark and Aalborg University, Aalborg, Denmark*) was used in the conditioning session to assess the cuff pressure pain and to induce a constant, painful pressure on the lower leg. The technique has previously been shown capable of activating endogenous pain inhibitory mechanisms in healthy individuals (Graven-Nielsen et al., 2017; Imai et al., 2016) and has been used to demonstrate reduced efficiency of endogenous inhibitory mechanisms in chronic pain patients compared with controls (Graven-Nielsen et al., 2015; Imai et al., 2016; McPhee and Graven-Nielsen 2019a; Petersen et al., 2019; Skou et al., 2013). A double-chamber cuff (*VBM, Sulz, Germany*) was placed on the lower leg on the non-dominant side, with the upper rim of the cuff being level with the head of fibula. For mechanosensitivity assessments, both chambers of the cuff were inflated gradually (1 kPa/s). The participant used an

electronic visual analogue scale (VAS) to rate the intensity of pressure pain. The VAS was anchored with 0 as 'no pain' and 10 cm as 'worst pain imaginable'. The pressure pain detection threshold (PDT) was defined as the pressure where the VAS score exceeded 1 cm the first time. The subject was asked to continuously rate the pain intensity on the VAS as the cuff pressure increased. When the pressure reached the level where it became intolerable (VAS 10/10), the participant was instructed to press a button that stopped the cuff pressure stimulation and the cuff was deflated. The pressure where the stimulation was stopped defined the pain tolerance threshold (PTT). The PTT was recorded twice, and the average value used for further analysis. In case the PTT was not reached before reaching the safety limit (100 kPa) of the cuff algometer, the PTT was defined as 100 kPa. To induce a tonic painful conditioning stimulus, the pressure cuff was inflated rapidly (100 kPa/s) until it reached a pressure level equivalent to 80% of PTT. This pressure was maintained while referred pain assessment protocol was induced (see description above) and the cuff was deflated immediately after.

Statistics

Parametric distributed data are presented as mean and standard errors of the mean (SEM) and non-parametric data as median and interquartile range [IQR, 0.25 – 0.75]. Distribution characteristics of data was determined by the Shapiro-Wilk test. Considering that as subset of healthy individuals do not develop referred pain from a nociceptive stimulus (Graven-Nielsen 2006), an analysis was done for the whole group but also separately based on the control session for those who developed referred pain and those who did not. Previous studies with group sizes of n=18 to n=20 have shown that a nociceptive stimulus to muscle tissue causes referred pain (symptoms outside the stimulation area) in approximately 60% of cases, which is further increased with tissue sensitization (Domenech-Garcia et al., 2016; Gibson et al., 2006a; Gibson et al., 2006b). Therefore, we expected that 15/24 subjects would demonstrate a reduction in pain referral during the conditioning session. A distinction was made between the groups based one whether pain was felt remote to the stimulation site (pain referral, according to the definition above) or not (no-pain referral).

For the whole group analysis, between-session comparisons were based on a paired t-test or a Wilcoxon test. All pairwise comparisons were Bonferroni corrected to account for multiple comparisons. A significance level of 0.05 was accepted.

RESULTS

All participants completed all parts of the study. Therefore, a full dataset from 24 male subjects (27 years (range 18-37)) was available for data analysis with 15 subjects (27 years (range 18-37)) reporting referred pain in the control session.

Baseline pressure-pain sensitivity at the infraspinatus muscle

No differences were found in baseline PPTs at the infraspinatus muscle when comparing the control and conditioning sessions (Table 1, $t(23) = 0.87$, P = 0.39**1**) or when comparing the referred pain group and non-referred pain groups within sessions and between groups (ANOVA: $F(1,1) = 0.41$, $P = 0.534$).

The pressure needed to induce pain at NRS7 was comparable between the control and conditioning sessions in the whole group ($t(23) = 0.484$, $P = 0.634$).

Cuff conditioning

No difference was found in PDT between the referred pain (29.8 ± 12.0 kPa) and the non-referred pain (31.0 ± 17.8 kPa) groups ($t(22) = -0.22$, P = 0.188). Likewise, no difference was found in PTT between the referred pain group (64.2 ± 18.6 kPa) and the non-referred pain (61.5 ± 20.2 kPa) groups ($t(22)= 0.34$, P = 0.747). Thus, the intensity of the painful remote stimulus was comparable between the two groups.

Pressure-induced referred pain

In general, pressure-induced pain was found at the stimulation site (local pain) and referred to the anterior and posterior shoulder/arm/hand (Fig. 1). Therefore the size of pain referral was extracted for both the front and back view drawings. **In general, the referred pain area was larger in the back view compared with the front view at both time points (control and conditioning) and also when analysing the total group or the referred pain and non-referred pain groups separately.** For the total group, the pain drawings at the back revealed a smaller pain area in the conditioning compared with the control session (Wilcoxon: $Z = 2.26$, $P = 0.024$), which was not significant for the front (Wilcoxon: $Z = 51$, $P = 0.079$). The sum of reported pain area from the front and back body charts was different between the control and

conditioning sessions with a smaller area in the conditioning session in the total group (Wilcoxon: $Z = 3.17$, $P = 0.003$, Table 2).

Pain areas were extracted where the changes between sessions represent differences in number of pixels drawn regardless of whether these were outside (referred-pain) or only within (non-referred pain) the stimulation area (table 2). Within the referred pain group, a reduction in size of the painful area in the back-view found in the conditioning session compared with the control session (Wilcoxon: $Z = 2.7$, $P = 0.009$), but not in the front-view of the body chart (Wilcoxon: $Z = 0.94$, $P = 0.683$). In the referred pain group, the total pain area (front + back) was smaller in the conditioning session, compared with the control session (Wilcoxon: $Z = 3.01$, $P = 0.005$, Table 2). None of the subjects in the referred pain group drew a pain area only within the stimulation area in the conditioning session. For the non-referred pain group, no significant difference was found between the two sessions in the front-view of the body chart (Wilcoxon: Z = 1.37, P = 0.341), at the back-view (Wilcoxon: $Z = 0.18$, $P = 0.852$), or in both (Wilcoxon: $Z = 1.13$, $P = 0.524$). Two subjects from the non-referred pain group drew areas of pain outside the stimulation area in the conditioning session.

Distribution of pressure-induced pain

No significant difference was found in the length of pain distribution between the control and conditioning sessions for the total group (n = 24) in the front or back view (Wilcoxon: $Z = -0.36$: $P = 0.361$). The referred pain group showed no significant difference between the control and conditioning sessions for the length of pain spread in the front-view of the body chart (Wilcoxon: $Z = -1.78$: $P = 0.152$) or the back (Wilcoxon: $Z = -2.04$: $P = 0.082$).

DISCUSSION

This is the first study to show a painful and remote conditioning stimulus reduces the area of referred pain originating from the location of the primary pain-evoking stimulus. These results provides further support for the mechanisms involved in referred pain and indicate that the distribution can be modulated by descending control systems.

Modifying the mechanisms of referred pain

In this study, referred pain was induced in the infraspinatus muscle as it is known that a painful stimulation of the shoulder blade muscles causes a large pain referral in healthy individuals (Domenech-Garcia et al., 2016; Leffler et al., 2000) as also confirmed by the present findings. However, stimulating other body areas may not result in a similarly extensive pain referral (Graven-Nielsen 2006), indicating that the current findings might be non-replicable in muscles predominately evoking local pain (e.g. biceps and triceps brachii muscles) in contract to other muscles with prominent pain referral (tibialis anterior and brachioradialis muscles)(Graven-Nielsen 2006).

Patients suffering from chronic musculoskeletal pain demonstrate expanded patterns of referred pain, as evoked from a standardized nociceptive stimulus and this is considered to be a result of facilitated central mechanisms (Graven-Nielsen and Arendt-Nielsen 2010; Kosek and Januszewska 2008; O'Neill et al., 2007). More recently, it has been shown that individuals who have recovered from a shoulder fracture or an ankle injury, demonstrate a facilitated pain referral (Domenech-Garcia et al., 2018; Palsson et al., 2018) which also underscores that central sensitization can persist in asymptomatic (pain-free) cases. This current study indicates that such a distribution can be reversed in healthy individuals. Reduced efficacy of endogenous pain inhibition, as observed in patients with chronic musculoskeletal pain (Christensen et al., 2020; Gerhardt et al., 2017; McPhee and Graven-Nielsen 2019b), are referenced as mechanisms contributing to widespread/multisite pain but not local pain (Gerhardt et al., 2017). However, administering ketamine, an NMDA antagonist, it is possible to reduce the area of pain referral in fibromyalgia patients (Graven-Nielsen et al., 2000) and healthy controls (Schulte et al., 2003), revealing the central nature of mechanisms underlying pain referral. Referred pain is likely a result of an enlargement in the receptive fields with an engagement of both nociceptive-specific and wide dynamic range neurons in laminae I-VI (Hoheisel et al., 1993). Moreover, mechanisms of descending modulation also terminate on laminae I/III, II and V/V1 (Patel and Dickenson 2020; Suzuki et al., 2002; Todd 2010), where wide dynamic range neurons are specifically modulated by endogenous pain inhibitory mechanisms (Guan et al., 2006). This overlap in mechanisms terminating on and thus potentially affecting the same wide dynamic range neurons therefore provides a potential neuroanatomical link that may explain the modulatory effects of pain referral in the present study.

The nervous system undergoes several changes following a nociceptive insult, both at cortical (De Martino et al., 2017; Schabrun et al., 2016), spinal (Gong et al., 2019; Kuner and Flor 2016) and peripheral level (Schaible et al., 2011). Previously, it has been suggested that the size of pain referral patterns could be a useful proxy for evaluating the sensitivity of central pain mechanisms (Domenech-Garcia et al., 2016; Graven-Nielsen and Arendt-Nielsen 2010). There are a number of limitations to this premise: the stimulation intensity is difficult to standardize with manual palpation but the size of referred pain areas is related with stimulation intensity (Arroyo-Fernandez et al., 2020; Palsson et al., 2020). Therefore, even though referred pain can be induced by manual stimulation (Schiffman et al., 2014), it would be unrealistic to believe that manual stimulation can be standardized across individuals and clinicians making the quantification of day-to-day differences unreliable. Another factor to consider is the variance in pain referral patterns between individuals as observed in this study and others (Boudreau et al., 2017; Boudreau et al., 2018; Domenech-Garcia et al., 2016; Gibson et al., 2006b; Palsson et al., 2018). Recently, it was demonstrated that variability in pain referral is greater with lower stimulation intensities (Palsson et al., 2020) where the optimal stimulation intensity, based on variability of pain referral, is above 30% of the PPT as also used in the present study (table 1). In the Palsson et al study, higher intensities did not reduce the coefficient of variance for pain referral additionally. This variability in a healthy system makes it difficult to determine the level of pain sensitivity on an individual patient level. Moreover, it is important to consider the spectrum of responses to a nociceptive conditioning stimulus where both healthy individuals (Firouzian et al., 2020) and people with chronic pain (Rabey et al., 2015; Vaegter and Graven-Nielsen 2016) can demonstrate either pro- and/or anti-nociceptive response to a standardized nociceptive stimulus even though they belong to the same group. Here, it is essential to consider that a range of cognitive and emotional factors can, via the endogenous inhibitory mechanisms, modulate the excitability of post-synaptic neurons (Bushnell et al., 2013).

Factors affecting referred pain

In this study, participants were asked to focus on the area of referred pain caused by a minute-long pressure stimulus. This was done with and without a competing nociceptive stimulus from the lower leg. In this case, it can be relevant to consider that acute pain negatively affects the

ability to perform tasks that require cognitive resources (Moore et al., 2019). It could therefore be argued that the reduction in pain area due to the painful conditioning stimulus (table 2) merely reflects a cognitive disturbance. The influence of attention on endogenous pain inhibition has however previously been questioned (Lautenbacher et al., 2007; Moont et al., 2010). Interestingly, recent evidence suggests that persons with a less efficient conditioned pain modulation response get an analgesic response from diverting the attention whereas this does not affect those with a better functioning system (Hoegh et al., 2019). This underlines that in a healthy system, individual differences, including general sensitivity of the pain system, can potentially explain why only 15 out 24 subjects experienced referred pain in this study. Such individual characteristics may likewise explain that two subjects from the non-referred pain group indicated referred pain in the conditioning session. The proportion of subjects developing referred pain is similar to what has previously been demonstrated in healthy subjects (Domenech-Garcia et al., 2018; Gibson et al., 2006a; Graven-Nielsen and Arendt-Nielsen 2010; Lei and You 2012).

In this study we set out to draw parallels between endogenous pain inhibition and the size of somatic referred pain. It is however important to acknowledge the potential role of segmental inhibition on the findings, especially considering that 40% of the participants did not experience referred pain. The mechanism is considered to involve activation of segmental wide-dynamic range dorsal horn neurons that, depending on their sensitivity may facilitate or attenuate afferent signals from the periphery (Hao et al., 1992; Yang et al., 2014). For clinical pain management, this mechanism is actively employed in spinal cord stimulation (Taylor et al., 2014). Although speculative, it is possible that the combined effect of descending endogenous and segmental inhibition is necessary to gain sufficient pain relief. An investigation hereof is warranted.

Nociceptive afferent input can be suppressed by cognitive and emotional processes; processes, that also involve endogenous pain modulating circuits (Bannister and Dickenson 2017; Bee and Dickenson 2009; Doan et al., 2015). Interestingly, a strong association seems to exist between pain referral and negative emotionality where an experimentally-induced referred pain area is larger in pain-free individuals with negative emotions (Lee et al., 2013). It is however unclear whether relationship exists between psychological factors and pain drawings reported by people living with chronic musculoskeletal pain (Reis et al., 2019). When evaluating the size and distribution of pain areas, it is therefore important to account for the complicated

interactions between nociceptive processes, cognitive and emotional factors and the endogenous inhibitory circuits.

Limitations and future steps for methodological refinement

When designing the study, the stimulation intensity required to induce NRS7 was expected to increase during the conditioning nociceptive stimulus applied the lower leg, similar to what has been demonstrated in other experimental pain studies (Graven-Nielsen et al., 2017; Imai et al., 2016). This did however, not occur most likely because the high stimulation intensity (NRS7) resulted in a ceiling effect with little or no space for an additional increase during the conditioning state. Adding a distant PPT site would allow an evaluation of the effectiveness of endogenous pain inhibition. Also, this would allow for an evaluation of a potential relationship between the effectiveness of endogenous pain inhibition and changes in the pain referral area and should therefore be recommended in future studies with a similar aim. **The pressure applied to induce NRS7 was adjusted approximately every 10 seconds if needed to ensure the relative pain intensity was similar for all participants throughout the sixty seconds. The pressure level after 60 seconds was extracted for data analysis but we did not record if/how often the pressure was adjusted during the stimulation. Although it is unclear whether this would have changed the outcome, it could have enhanced the accuracy if we could have used e.g. the average pressure stimulation value instead of the final one.**

The effect of the conditioning is related to the conditioning intensity, where a more intense conditioning stimulus results in a greater descending inhibitory response (Arendt-Nielsen et al., 2015; Graven-Nielsen et al., 2017; Kennedy et al., 2016). In this study, the two stimuli were relatively comparable, i.e. 80% of PTT for the pressure cuff and NRS7 for the handheld algometer and applied simultaneously in the conditioning session. However, it must be acknowledged that continuous monitoring of the stimulation was only possible with the handheld pressure algometer. Therefore, we cannot fully know if the PTT (and thereby also 80%PTT) changed during the 60 seconds. Once engaged, the activity of endogenous pain inhibitory mechanisms can remain in a facilitated state 3-5 minutes (Graven-Nielsen et al., 1998; Lewis et al., 2012), gradually fading over time (Pud et al., 2009). Therefore, to circumvent the bi-directional nature of the two stimuli used here, assessing the pain referral immediately after the cuff pressure was relieved could have been possible.

The inclusion of only male participants in this study may underrepresent the occurrence of referred pain in the general population as females show a higher frequency of experimentally evoked referred pain (Frey Law et al., 2008; Graven-Nielsen 2006). **Generalizing the study findings should be done with care, considering the small sample size and large variability in pain referral. A similar study with a larger sample, including males and females is warranted.**

The influence of cognitive and emotional factors as well as sleep on these results were not assessed. Indeed, it is known these factors can influence pain referral patterns and the efficiency of endogenous inhibition in healthy subjects (Lee et al., 2013; Smith et al., 2007; Weissman-Fogel et al., 2008). Controlling for these factors could be relevant in a study like this to better understand individual responses to the test protocol.

Pain area was expressed in pixels where two individuals could report e.g. local pain around the stimulation site or pain in the arm or forearm. In these two examples, the number of pixels could be similar despite clear distinction between the pain distributions where the first one would not fulfil the criteria for referred pain used in this study. We defined referred pain as all pain reported outside the boundaries of the scapula. We considered this approach to be more conservative than considering local pain in the scapular area to be referred pain. By using the borders of the scapula as a reference point, we likewise consider that an element of subjectivity was reduced in the interpretation of what constitutes referred pain.

Conclusion

Engaging the descending noxious inhibitory control reduced the size of pain areas evoked by mechanical stimulus, predominately when distinct pain referral was present. Assuming a conditioning effect due to descending inhibitory control acting on dorsal horn neurons, these findings suggest that mechanisms underlying pain referral can be modulated by endogenous control. Future investigations on relationships between the effectiveness of endogenous inhibition and pain referral patterns in humans are warranted.

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Figure legend

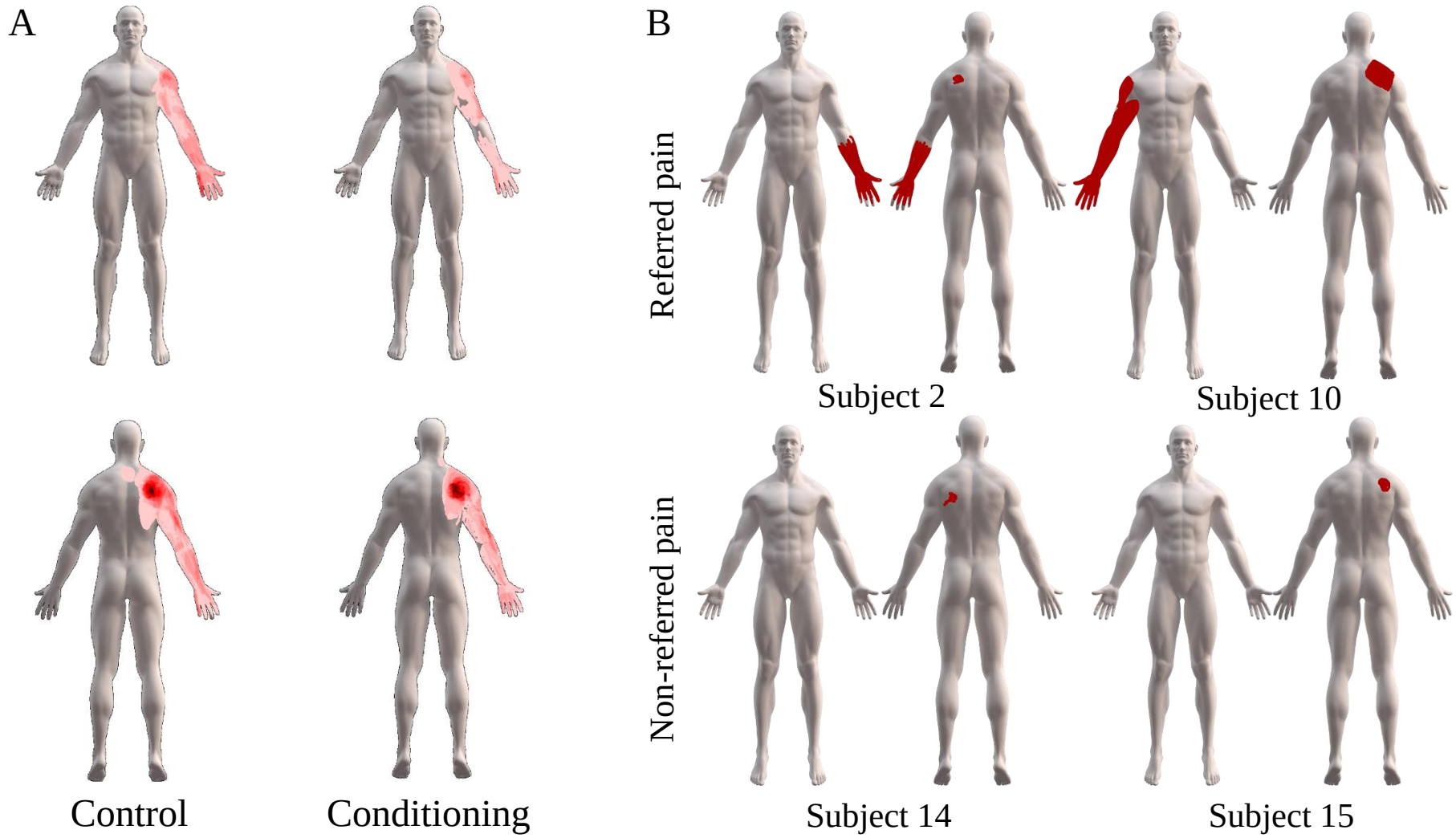
Figure 1 (A) Pain referral patterns during the control session (left side) and the conditioning session (right side). Going from dark to light, the colour codes indicate the proportion of subjects (n =24) who experienced pain or referred pain in the area. (B) Example of pain distribution in two subjects from the referred pain group (above) and two subjects from the non-referred pain group (below).

	Pressure thresholds (kPa)			Stimulation intensity (kPa)		
Group	Control session	Conditioning session		Control session	Conditioning session	
	PPT	PPT	**PTT**	NRS7	NRS7	**80%PTT**
Total (n=24)	402.4 ± 183.9	385.6 ± 160.3	**63.2 ± 3.8**	689.5 ± 255.6 **(39.9% over PPT)**	673.5 ± 291.2 **(40.5% over PPT)**	**50.5 ± 3.1**
Referred pain (n=15)	374.1 ± 172.6	347.7 ± 134.5	**64.2 ± 19.0**	686.8 ± 254.8 **(44.2% over PPT)**	652.3 ± 294.9 **(43.6% over PPT)**	**51.3 ± 15.2**
Non-referred pain (n=9)	449.5 ± 182.2	448.7 ± 170.8	**61.5 ± 19.1**	693.9 ± 242.5 **(33.0% over PPT)**	708.9 ± 264.1 **(35.2% over PPT)**	**49.2 ± 15.2**

Table 1. Mean (± SD) baseline pressure intensity needed for the pressure pain threshold ***(PPT), pain tolerance threshold (PTT),*** *pressure pain intensity equivalent to 7 out of 10 on the numeric rating scale (NRS7)* ***and the conditioning stimulation intensity (80%PTT)*** *for the total, referred pain and non-referred pain groups.*

	Front view		Back view		Sum of front and back view	
Group	**Control**	**Conditioning**	**Control**	**Conditioning**	**Control**	**Conditioning**
Total (n=24)	5882 [0 - 19199]	3840 [416-11643]	14133 [5202 - 26577]	8119* [3335 - 17497]	23398 [10631 - 47796]	15865** [7044 - 26115]
Referred pain (n=15)	8400 [3033 - 19984]	6144 [2490 -12690]	23379 [14733-41567]	9825# [4623-18295]	34124 [19034 - 59049]	21255## [13253- 31079]
Non-referred pain (n=9)	0 [0 - 0]	1774 [0 - 3634]	5160 [3493 - 7277]	3296 [2659 - 9119]	5160 [3493 - 7277]	7118 [4983 -16287]

Table 2. Median [IQR] size of pain area in pixels following a painful stimulus to the infraspinatus muscle in the control and conditioning sessions. Pain areas are shown for the front and back as well as the sum of back and front for the total, referred pain, and non-referred pain groups. Significant difference compared with the control session (, P* ***= 0.045; #, P = 0.011; **, P = 0.003; ##, P = 0.005****).*


A
B
Referred pain
Non-referred pain
Subject 2
Subject 10
Subject 14
Subject 15
Control
Conditioning