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Tempo-spatial integration of nociceptive stimuli assessed via the nociceptive withdrawal reflex in healthy humans.

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Running head: Spinal tempo-spatial integration of nociception via the NWR

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ABSTRACT

Spatial information of nociceptive stimuli applied in the skin of healthy humans is integrated in the spinal cord to determine the appropriate withdrawal reflex response. Double-simultaneous stimulus applied in different skin sites are integrated, eliciting a larger reflex response. The temporal characteristics of the stimuli also modulate the reflex e.g. by temporal summation. The primary aim of this study was to investigate how the combined tempo-spatial aspects of two stimuli are integrated in the nociceptive system. This was investigated by delivering single and double simultaneous stimulation, and sequential stimulation with different inter-stimulus intervals (ISIs ranging 30-500 ms.) to the sole of the foot of fifteen healthy subjects. The primary outcome measure was the size of the nociceptive withdrawal reflex (NWR) recorded from the Tibialis Anterior (TA) and Biceps Femoris (BF) muscles. Pain intensity was measured using an NRS scale. Results showed spatial summation in both TA and BF when delivering simultaneous stimulation. Simultaneous stimulation provoked larger reflexes than sequential stimulation in TA, but not in BF. Larger ISIs elicited significantly larger reflexes in TA, while the opposite pattern occurred in BF. This differential modulation between proximal and distal muscles suggests the presence of spinal circuits eliciting a functional reflex response based on the specific tempo-spatial characteristics of a noxious stimulus. No modulation was observed in pain intensity ratings across ISIs. Absence of modulation in the pain intensity ratings argues for an integrative mechanism located within the spinal cord governed by a need for efficient withdrawal from a potentially harmful stimulus.

NEW & NOTEWORTHY

Tempo-spatial integration of electrical noxious stimuli was studied using the nociceptive withdrawal reflex and a perceived intensity. Tibialis Anterior and Biceps Femoris muscles were differentially modulated by the temporal characteristics of the stimuli and stimulated sites. These findings suggest that spinal neurons are playing an important role in the tempo-spatial integration of nociceptive information, leading to a reflex

response which is distributed across multiple spinal cord segments and governed by an efficient defensive withdrawal strategy.

INTRODUCTION

The NWR was originally described as an elicited motor response characterized by a stereotyped flexion of one or several joints aiming at removing the limb from a potentially tissue damaging stimulus (1). The withdrawal was interpreted as the activation of synergistic muscles to produce flexion of the exposed limb, including the inhibition of antagonist muscles and a compensating opposite process in the contralateral limb to preserve balance (1). More recently, evidence has suggested a functional reflex organization in which the NWR involves flexion and extension of relevant joints, by recruiting multiple groups of muscles, depending on the site being stimulated (2–6).

Spatial integration of the nociceptive stimuli into a reflex response might involve processing in supraspinal centers. Primary afferents that depolarize due to a nociceptive stimulus will first synapse in the dorsal horn of the spinal cord. Further processing likely happens through a complex network of inhibitory and excitatory interneurons within the dorsal spinal cord(7). Then, ascending information is projected via the anterolateral system to supraspinal structures (brainstem, thalamus and cortex) eventually producing the perception of pain(8). In parallel, information transmitted to the ventral horn of the spinal cord can lead to the elicitation of the protective NWR. Neurons descending from superior structures (primarily the rostroventral medulla in the brainstem) synapsing in the dorsal horn of the spinal cord have been reported (see 9 for a review) and form the basis of the supraspinal descending excitatory and inhibitory modulation maintaining homeostasis of the spinal nociceptive processing.

Previous evidence from animal studies suggested that deep dorsal horn neurons encode the organization of the NWR in modules that allow the coordination of muscle recruitment to generate optimal withdrawal (10–13). Human studies have also supported the presence of this spinal modular organization describing the NWR patterns elicited by stimulation in several sites of the sole of the foot (2). In this context, the term Reflex Receptive Field (RRF) was implemented to define the area of the skin from which a suprathreshold stimulus triggers the recruitment of a certain muscle or group of synergistic muscles (2, 5). The RRFs described in the sole of the foot of healthy humans suggest that the NWR pathway integrate spatial information of the stimulus to elaborate an optimal defensive response. It remains unknown, to what degree this spatial integration is altered when introducing a secondary stimulus shortly after the first. It is known that both the spatial characteristics of the stimulus modulate the NWR, but so does the temporal characteristics. By delivering repetitive electrical stimulation to the same site on the sole of the foot, several studies have shown that temporal summation can modulate the NWR. The magnitude of the NWR is significantly increased with repetitive input (14–20), showing a reflex behavior that resembles the wind-up phenomenon observed in single neuronal recordings in animal studies (21–23). To what extent the temporal summation can be translated into tempo-spatial integration has not been studied. By clarifying this, it may shed light on the spinal tempo-spatial integration of nociceptive input.

As recently shown in healthy humans, double stimulation to different skin sites is indeed integrated, most likely at spinal level, as shown by increased NWR magnitudes for double stimulation compared to single stimulation (24). Moreover, the inter-electrode-distance modulated the magnitude of the NWR, suggesting that spinal cord neurons do integrate spatial information (24). The use of double stimulation in the same or different sites of the sole of the foot, and with varying inter-stimulus-intervals (ISIs), may provide valuable information about spinal integration of nociception. The temporal discrimination (TD) of two sequential stimuli, can also be used to investigate how nociceptive temporal information is processed in the somatosensory system (25–29). TD likely depends on multiple stages of integration at spinal and supraspinal levels (28, 30). TD for innocuous and noxious stimuli have been reported using stimuli of different nature (laser, electricity and mechanical). Although TD may be representing purely temporal processes of integration, it is unknown if TD also incorporates spatial information in the perception (26).

The present study aimed at assessing the modulatory effect of a temporal delay between two stimuli delivered to the same or to two different sites in the sole of the foot in healthy humans. By using simultaneous and sequential electrical stimulation with different Inter-Stimulus Intervals (ISIs), tempo-spatial integration of nociceptive information in the spinal pathways was studied. It was hypothesized that shorter ISIs are processed as sensory input with higher potential to produce damage and therefore associated with a facilitation of the NWR. Spatial summation of the NWR when using double simultaneous stimulation was also expected. The integration was primarily probed by estimating the magnitude of the NWR and psychophysical measures were obtained as secondary outcomes. Temporal discrimination thresholds (TDT) of the nociceptive system were also examined as an exploratory outcome.

METHODS

Participants

Fifteen healthy human subjects (9 men and 6 women, between 22 and 31 years old) participated in the study. Prior to the experimental session, oral and written information was given to participants. If the subjects agreed to participate, written informed consents were obtained prior to the experiment. Exclusion criteria included any use of medication with known effects on the CNS that could influence the results of the study. In addition, volunteers were prevented to participate in case of pregnancy or breastfeeding, previous mental, musculoskeletal or nervous disorders, presence of chronic or acute pain, skin wounds on the site of stimulation, or inability to cooperate during the experimental session.

The study was approved by the local Ethics Committee (North Denmark Region; VN-20180047) and conducted in compliance with the Declaration of Helsinki.

Electrical Stimulation

To elicit the NWR, electrical stimulation was applied in the sole of the right foot. The skin was prepared by manual abrasion of the sole of the foot to reduce skin impedance due to the thick stratum corneum. Two stimulation electrodes (Neuroline 700, Ambu A/S, Denmark, reduced to a diameter of 6 mm (24, 31)) were mounted on the skin over the first and fifth metatarsal bone in the sole of the foot (medially (M) and laterally (L) located, respectively; Figure 1A). A large anode (7.5x10 cm; Pals, Axelgaard Ltd., Fallbrook, California, USA) was placed on the dorsum of the foot for the stimulation to be perceived in the sole of the foot (31).

-Please, insert Figure 1-

Three types of stimuli were delivered: single, simultaneous, and sequential. Single stimulation through either electrode (M or L), simultaneous stimulation through both M and L electrodes, and sequential stimulation (through the same electrode: M or L; and as a combination of both: M, then L). Sequential stimulation was delivered with varying ISIs: 30 ms, 50 ms, 100 ms, 150 ms, 200 ms and 500 ms (2). Thus, in total 21 different configurations of stimuli were delivered during the experimental session. An overall interstimulus rate of 20-30 s (randomized) was used to reduce habituation throughout the experiment (32).

-Please, insert Figure 2-

Two constant current computer-controlled electrical stimulators (Noxitest IES 230, Aalborg University, Denmark), were used to stimulate each electrode (M and L), allowing individual control of the stimulation intensity (St_i) in each electrode. St_i was individually set for M and L based on the pain threshold (P_{th}) and the NWR threshold (NWR_{th}), and St_i was defined as the lowest intensity that simultaneously satisfied the following conditions:

$$St_i > 1.2 \times P_{th} \text{ AND } St_i > 1.2 \times NWR_{th}. \qquad \text{Equation 1}$$

A staircase procedure was performed to estimate P_{th} and NWR_{th} for each stimulation electrode, following an automated procedure described in a previous study (33). The criterion for P_{th} estimation was a rating of perceived pain intensity above threshold (NRS=5) and for the NWR_{th}, a detection of a NWR when the interval peak z-score of the recorded signal over the reflex windows exceeded a value of 12 (34).

The initial stimulation intensity was 1 mA and it was increased in steps of 2 mA until the criteria for the estimation was detected (Equation 1). Subsequently, intensity was decreased by steps of 1 mA / 0.5 mA (NWR_{th}/P_{th}) until the criteria was no longer met. Increasing and decreasing steps of 0.5 mA / 0.1 mA (NWR_{th}/P_{th}) were then used and the thresholds were calculated averaging the last three peaks and troughs derived from this process. The experiment was terminated if stimulation current reached 50 mA before the criteria above (Equation 1) was met.

Electromyography

Surface electromyography of Tibialis Anterior (TA) and Biceps Femoris (BF) muscles was recorded on the right leg (Figure 1B). The skin was shaved to remove hair growth and reduce impedance due to the stratum corneum. Double differential configuration (35) was used by mounting three recording electrodes (Neuroline 720, Ambu A/S, Denmark) following SENIAM recommendations on electrode location for TA and BF (36). A common reference electrode (Neuroline 720, Ambu A/S, Denmark) was placed at the patella of the ipsilateral knee. Recordings were amplified, bandpass-filtered (5-500 Hz), sampled (2 kHz), and stored for offline analysis.

Experimental protocol

Subjects were lying supine on a reclined bed with their legs relaxed. The experimental session started with a familiarization protocol in which a series of stimuli were delivered in both stimulation sites (M and L) in random order with varying intensities. The aim of the familiarization block was to introduce the participant to the electrical stimuli and the rating tasks, and to reduce potential effects of anxiety and arousal. In the second block P_{th} and NWR_{th} were estimated as previously described. During the third block, single stimulation, simultaneous stimulation, and sequential stimulations were delivered (Figure 2) with five repetitions of each condition in random order (21 conditions, 105 stimulations in total). Rest periods of 3-5 minutes duration were taken after every 25 stimulations. After each stimulation in block three, subjects were asked to report an overall rating of the intensity of the perception, using a Numerical Rating Scale (NRS) anchored at 0 with the label "No perception", at 5 with "Pain threshold", and at 10 with "Worst pain imaginable", and to indicate the stimulated site(s).

Data analyses and statistics

Magnitude of the NWR

EMG signals were filtered offline (5-500 Hz) and visually inspected to detect possible artifacts and outliers. The root mean square (RMS) was calculated in the reflex window defined as 80-150ms post stimulus (2) (Figure 2). Signals were then averaged across the five repetitions for each condition. Recordings were excluded in case of abnormally high amplitude of both TA and BF signals, based on a robust estimation method using a MAD-Median rule (37).

The averaged NWR due to sequential stimulation in the same electrode (M or L) was then normalized by dividing it with the averaged NWR due to single stimulation in the respective electrode (M or L). For simultaneous stimulation, the average of the responses to single stimulation in both sites (M and L) was used to normalize. Therefore, the reported values (nNWR) express as a ratio between the NWR under a specific stimulation condition (simultaneous or sequential) and the corresponding response due to single stimulation in the respective site(s).

Temporal discrimination threshold (TDT)

To calculate the TDT, responses were classified as being perceived as a single or double stimulus. Stimuli perceived as single were coded as ”0” while stimuli perceived as double were coded as "1". Then, the responses due to the five repetitions of the same stimulus were averaged. A logistic regression was used to estimate TDT (see Equation 2 bellow) (Frahm et al., 2018; Mørch et al., 2010; Schlereth et al., 2001).

Parameter “b” indicates the ISI for which participants were able to discriminate the two stimuli ($y=0.5$ in Equation 2), defined as the Temporal Discrimination Threshold (TDT). The parameter ‘a’ indicates the slope of the sigmoidal curve at x = ‘b’.

$$y = \frac{1}{1+e^{(a(b-x))}} \qquad \text{Equation 2}$$

Statistics

Normality of data was previously assessed in all data sets (Shapiro-Wilk test) to choose between parametric or non-parametric statistical tests. Repeated measures analyses of variance (RM-ANOVA) was used when normality and sphericity (Mauchly test) was confirmed, otherwise, Friedman’s test was used.

Friedman’s test was used to assess the effect of the ISI on the magnitude of the NWR. As differences between adjacent ISIs were not of interest according to the hypotheses, post hoc analyses focused on the comparison of the two extreme conditions on both sides (30 ms, 50 ms vs 200 ms, 500 ms). Planned paired comparisons were conducted accordingly.

To assess the presence of spatial summation, RM-ANOVA was performed for pain intensity ratings during single stimulation in the medial sole of the foot (M), in the lateral sole of the foot (L), and double simultaneous stimulation in both sites (M and L). NRS ratings were averaged across all five repetitions of each condition before conducting the statistical tests.

To compare the TDTs based on the spatial characteristics of the stimuli (delivered in M, L or both sites), the 95 % confidence intervals of the estimated thresholds were analyzed to assess significant differences.

When significant main effects were found, posthoc analysis with adjusted multiple comparison was conducted. For non-parametric data, Wilcoxon signed rank test was performed with Bonferroni-Holm adjustment for multiple comparison. When using RM-ANOVA, Bonferroni-Holm posthoc analysis was preferred to adjust for multiple comparisons. P-values smaller than 0.05 were considered significant.

RESULTS

Magnitude of the NWR

Compared to single stimulation, simultaneous stimulus provoked significantly larger reflexes in both TA and BF, suggesting the presence of spatial summation in the NWR (Figure 3, ø: $p < 0.01$). Simultaneous stimulation elicited larger reflexes than sequential stimulation in TA (Figure 3, *: $p < 0.05$), no statistically significant difference was found in BF (see raw EMG traces of a representative subject in Figure 1C). On the other hand, sequential stimulation (averaged across all ISIs) only elicited larger reflexes in the BF muscle.

-Please, insert Figure 3-

NWR for sequential stimulation with varying ISIs

During sequential stimulation, a main effect of ISI on the TA-NWR was found regardless of the stimulated site (M: Figure 4 top row, left column $p < 0.001$; L: Figure 4 top row, center column $p < 0.01$ and the combination M-L: Figure 4 top row, right column $p < 0.05$). Stimulation with longer ISIs elicited larger reflexes in TA, regardless of the stimulated site (M, L, and their combination; Figure 4 top row *****$p < 0.01$).

For the BF muscle, a significant main effect of ISI was found when stimulating through the L electrode (Figure 4 bottom row, center column $p < 0.05$) and in the combination of both M and L (Figure 4 bottom row, right column $p < 0.001$). When stimulating through the M electrode, BF-NWR showed a similar tendency towards smaller reflexes for longer ISIs (Figure 4 bottom row, left column $p = 0.06$). Results of post hoc analysis showed that longer ISIs produced decreasing NWR magnitudes in BF (Figure 4 bottom row *****$p < 0.05$).

-Please, insert Figure 4-

Perceived intensity

The mean perceived intensity responses to all stimuli were perceived above pain threshold (NRS=5), regardless of stimulated site or condition (single, simultaneous, or sequential).

As shown in Figure 5, the pain intensity following sequential and simultaneous stimulation were perceived as more painful than single stimulation ($p < 0.05$, Wilcoxon signed rank test), however, no difference was found between simultaneous and sequential stimulation.

-Please, insert Figure 5-

For sequential stimulation with different ISIs, regardless of the electrode site, there was no significant effect of the ISI ($p > 0.05$, Friedman's test) (Figure 6).

-Please, insert Figure 6-

Temporal discrimination thresholds

The estimated temporal discrimination thresholds (TDTs) are summarized in Table 1. The TDTs were 84.1 ms for the sequential stimulation in the medial site (M), 95.5 ms for stimulation in the lateral electrode (L); and 71.0 ms for stimulation as a combination of both electrode sites (M-L). The comparison between conditions showed that significant lower TDT was observed when stimulating both sites (M-L), rather than any single site. Additionally, TDT was lower for stimulation in the medial electrode (M) compared to the lateral electrode (L).

Table 1: Results from the logistic curve fitting to Equation 2. Results expressed as mean of each stimulation type with 95% Confidence intervals of the logistic regression.

Stimulation Type	TDT [ms]	95% CI [ms]
Medial electrode (M)	84.1	80.9-87.1
Lateral electrode (L)	95.5	93.5-97.6
Combination (M-L)	71.0	69.4-74.2

DISCUSSION

The current study investigated the tempo-spatial integration of double stimulation in healthy humans by assessing the magnitude of the NWR in one proximal and one distal muscle (BF and TA, respectively) during different stimulation conditions. Spatial summation was found in both spinal- (BF-NWR, TA-NWR) and supraspinal- (perceived intensities) mediated outcomes. Sequential stimulation with varying ISIs (between 30-500 ms) had a significant modulatory effect on the magnitude of the NWR but not on the perceived intensities. Interestingly, the modulation affected proximal (TA) and distal (BF) muscles differentially. For increasing ISIs, the TA-NWR were facilitated, while the opposite pattern was seen in the BF muscle. The temporal discrimination threshold was lower for stimuli delivered at different sites, compared to a single stimulation site.

Tempo-spatial integration in spinal nociception assessed via the NWR

Simultaneous double stimulation elicited a significantly larger NWR than single stimulation in both TA and BF suggesting that the spatial characteristics of the stimulation modulates the magnitude of the NWR (EMG traces of a representative subject in Figure 1C). When using simultaneous stimulation, the elicited NWR had an average magnitude of 150 % of the single stimuli (Figure 3). This observation is likely reflecting sub-additive spatial summation (SS) of the NWR in healthy humans. Evidence regarding SS in the somatosensory system is abundant in the literature, particularly in studies that used the intensity of the perception as an outcome of sensory integration (38–47). The NWR, being a polysynaptic reflex that reflects spinal nociceptive processing (48, 49), has not been explored for concurrent stimuli at different locations, except for one previous study (24). Our previous study and the current study show that simultaneous stimulation at two sites located medially and laterally in the sole of the foot (Figure 1A) elicits a NWR approximately 1.5 times larger than when stimulating the individual sites. These results support the notion that spinal cord neurons integrate afferent information from nociceptive fibers innervating skin regions in different dermatomes (40, 50, 51). From a defensive point of view, it is crucial to exploit this information since it allows an adaption of the reflex magnitude according to the spatial characteristics of the stimuli.

The difference in the NWR amplitude across varying ISIs, show that the temporal characteristics of the stimuli also play an important role in the modulation of the NWR (Figure 4). With increasing ISIs, the magnitude of the TA-NWR increased, while the BF-NWR decreased. Interestingly, this modulation seems to be independent of the stimulation site since the same tendencies were observed during stimulation of the medial electrode (M), the lateral electrode (L), and with the combination of electrodes (M-L) (Figure 4).

One possible explanation for the TA facilitation for larger ISIs (Figure 4; top row), could be varying degrees of temporal summation (TS) modulating the magnitude of the reflex. Modulation of the NWR due to TS, has previously been demonstrated (16, 17, 20, 52), however, the current study is the first to investigate TS in proximal and distal muscles concurrently when delivering stimulations in two different locations with different ISIs. A few human studies have compared the effect of the stimulus ISI on TS (16, 19, 52). Those studies showed that shorter ISIs generally produce larger TS effect. Although those observations seem to contradict the present ones, it is worth noting that the assessment of the magnitude of the NWR in previous studies was solely performed on proximal muscles: Semitendinosus (52), BF (16, 19) and Rectus Femoris muscles (19). Thus, those prior findings are consistent with the present results regarding the BF-NWR modulation. Comparing the modulation of TA-NWR and BF-NWR, it appears that the NWR modulation for varying ISIs differs in proximal and distal muscles consistently across stimulation sites. Thus, it seems plausible that the differential modulation on TA and BF reflect the protective role of the NWR. A sequential stimulation with shorter ISI is likely associated with afferent input with higher potential for damaging the body, regardless of the specific stimulated site on the foot. The optimal motor response to a more dangerous stimulus would be to generate a strong withdrawal of the entire limb, by preferentially recruiting proximal muscles. Stimuli with longer ISIs, on the other hand, do not seem to trigger such facilitated reflex magnitudes in the proximal muscles, suggesting that the recruitment of distal muscles (TA) could produce a sufficient withdrawal through dorsiflexion of the foot.

Interestingly, the temporal discrimination thresholds, suggest that stimuli with ISIs shorter than 70 ms are discriminated correctly in less than 50 % of all cases, regardless of the site being stimulated (see TDT in Table 1). In these cases, a crude withdrawal of the limb (by contraction of proximal muscles, e.g.: BF) seems to be the safest and optimal behavioral response to that noxious threat, rather than a more refined foot withdrawal (TA) (Figure 4).

In TA it was found that sequential stimuli produced significantly smaller reflexes compared to simultaneous stimuli (Figure 3). The spatial summation phenomenon that integrates simultaneous stimulation of M and L seems to diminish when the simultaneity is lost. This observation further argues for a dynamic tempo-spatial integration of nociception present in the spinal cord circuitry governing the NWR (2, 6, 11).

In summary, it appears that when delivering sequential stimulation, the nociceptive afferent volley is conveying temporal information, which is integrated within spinal cord reflex circuitry to elicit an optimal reflex response. With an ISIs short enough to prevent sensory discrimination as independent stimuli, the NWR circuitry seems to be responding as if the limb was exposed to a bigger threat (Figure 4) facilitating recruitment of proximal muscles (BF).

Descending modulation of the NWR

Evidence of supraspinal structures modulating motor defensive behavior have previously been reported (53–56). Specifically, the midbrain periaqueductal gray (PAG) likely play an important role in sensorimotor integration and exerts modulation over the motor system to assist survival (57). In the present study, particularly when using long ISIs (200-500 ms), the time between the first and second stimulus is sufficiently large to see top-down modulation of the NWR (58) triggered by the first stimulus (the NWR is reported as the size of the last NWR when using sequential stimulation, see Figure 2).

Moreover, it has been shown that different emotional states can modulate the magnitude of the NWR (59–62), which is outlining a pathway from cognitive processes to up/down regulation of the excitability in the spinal reflex pathways. Although preventive measures were taken in this study to familiarize the participants to the stimulation protocol, a top-down modulation of the NWR ascribed to the cognitive state cannot be completely discarded. Experiments involving painful stimulation, may induce a state of anxiety that can modulate spinal nociception (62). Evidence has recently been reported showing facilitation of the TA-NWR when the subject is exposed to an anxiety induced stimulation paradigm involving heterotopic painful electrical stimulation (62). Thus, it is interesting that the magnitude of TA-NWR was significantly larger for larger ISIs, which might involve a transcortical loop. However, if anxiety affected the subjects, the facilitation of spinal nociception would most likely affect the magnitude of the NWR for all ISIs. Additionally, the magnitude of the BF-NWR was significantly reduced with longer ISIs, suggesting that the modulatory effect of ISIs on the NWR magnitudes cannot be explained by changes in the cognitive state exerting spinal modulation.

The subjects' attention to the stimulus may induce inhibition of the NWR (63). Hence, in the present study, the first stimulus may draw the attention of the subject leading to smaller reflexes with the second stimulus. However, since stimuli were totally randomized and subjects were instructed to identify stimulated site in every trial, it seems unlikely that this effect is restricted only to some ISIs.

It is not known to what extent descending modulation differentially modulate proximal vs distal muscles. A recent study, however, has suggested that the unpredictability of the onset of an imminent stimulus produced differential modulation of proximal vs distal muscles involved in the NWR (64). In that study, when the onset of a second incoming stimulus (with an ISI of 1 s) was unknown (as in our study), the size of the NWR was differentially modulated with larger TA-NWR in combination with smaller BF-NWR. This is in agreement with the present results since the first stimulus may serve as a cue, particularly in the conditions with larger ISIs. This further support the NWR as a dynamic and highly adaptable behavioral response that integrates temporal and spatial information of the stimulus to produce optimal withdrawal.

Perceived intensities

Higher perceived intensity ratings were found for simultaneous and sequential compared to single stimulation, most likely caused by spatial summation (Figure 5). Although the exact mechanisms behind the summation phenomenon remains to be clarified, previous findings in animal studies and indirect evidence in human studies have shown that deep dorsal horn neurons likely play a major role in this central integration (38–40, 42, 65). From the present results, the fact that stimulated areas on the medial and lateral parts of the sole of the foot belong to different dermatomes (66, 67), likely support central neural circuits being responsible of the observed spatial summation in the perceived intensities.

However, it is important to notice, that although statistically significant, the average difference between simultaneous (and sequential) versus single stimulation in the perceived intensity of pain was less than half a point on the NRS, a similar difference in pain intensity as reported in other studies assessing SSP (40, 43, 46).

Contrary to the NWR modulation previously discussed, different ISIs did not modulate the perceived intensities (Figure 6). If shorter ISIs were indeed encoded as sensory input with more potential to produce damage, larger perceived intensity ratings may also be expected. However, this was not observed in this study (Figure 6). One reason that could explain this apparent disagreement between the reflex behavior and the intensity of the perception is that a larger number of stimuli are needed to establish a significant modulation in the perceived intensity due to temporal summation (68). Indeed, a previous study on temporal summation of pain and spinal nociception have shown that a significant modulation of pain perception is achieved when the skin is repetitively stimulated only after the delivery of the third stimulus with an ISI of 500 ms (69). The NWR responses observed in this study is affected by temporal summation which is already seen following the second stimulus (17), suggesting a larger reflex sensitivity to repetitive stimulation, while a larger number of pulses is needed to produce a modulation of the perceived intensity.

Temporal discrimination

Previous observations differ regarding the tempo-spatial discriminatory capabilities of the somatosensory system for noxious and innocuous stimuli. Specifically, better directional discrimination for noxious vs innocuous laser stimulation has been reported (70). However, when comparing noxious laser stimuli against innocuous mechanical stimulation the opposite was found (e.g. better discrimination for innocuous stimulation) (71, 72). The TDT in the present study (noxious) ranged between 71.0 ms and 95.5 ms (Table 1). For innocuous electrical stimulation in the lower limb, TD has been reported between 39.9 ms and 41.2 ms (25). A possible reason for the disagreement between the studies is likely the stimulation modality (laser, mechanical and electrical), which will affect the discrimination (73). Laser stimulation leads to a natural activation of nociceptors while electrical stimuli recruit a mix of Aδ and Aβ fibers, by bypassing the transduction processes and are associated with a high level of synchronization in the afferent volleys. Thus, temporal discrimination will also depend on the stimulation modality, and this may explain the disagreement between previous studies and the current study.

The TDT depends on the stimulation sites (M and L), for these two sites it is possible that different stratum corneum (SC) thicknesses explain the better improved temporal discrimination when the stimuli are applied in the medial side (thinner SC). Interestingly, the inclusion of a spatial factor (i.e. combination of M and L), in which the first stimulus is delivered in the medial side and the second stimulus in the lateral side of the sole of the foot, significantly improved the temporal discrimination of the stimulus (Table 1), arguing that spatial information is integrated and the perception reflects the temporal characteristics of nociceptive information.

Limitations

The NWR is a response elicited to defend the body from a potentially damaging stimulus. Together with this reflex response, the perception of pain may be elicited by the stimulus. The processing of the noxious stimulus that produces a defensive reflex response and a perceptual experience of pain involves several structures from the afferent fibers through the spinal cord and to the brain. In the present study, electrical stimulation was used to elicit the NWR, potentially depolarizing unmyelinated as well as myelinated fibers. Overall, a contribution of large myelinated fibers in the elicitation of the NWR cannot be completely discarded (74, 75). Given the duration of the recording window for the NWR, a contribution from supraspinal centers is likely. Previous studies have suggested that deep dorsal horn neurons encode spatial integration of nociception and do not project to ascending pathways (10, 50, 76). Likely arguing for spinal circuitry responsible, at least to some extent, for spatial integration and elicitation of the reflex response. On the other hand, perception involves many supraspinal structures to produce the perception of pain. A clear separation and independent assessment of both processes is, therefore, not possible in human studies.

Conclusion

The NWR circuitry is integrating the spatial characteristics of a sensory input, e.g. observed as larger reflex responses in both TA and BF when simultaneously stimulating two sites (compared to single stimulation; Figure 3). A similar phenomenon of summation was observed for perceived intensities (Figure 5). Moreover, TS of the NWR for sequential stimulation was found in both TA and BF muscles, and when the same and different sites were stimulated (Figure 4). TS was largest for small ISIs in the BF muscle, while the opposite pattern was observed in TA (larger TS for longer ISI). The perceived intensities were not modulated by varying ISIs (Figure 6). The sensory information concerning the temporal and spatial aspects of the stimulation shapes both the reflex response and the perception in terms of temporal discrimination. The different tendencies in the modulation observed in the TA and BF muscles for different ISIs and for different stimulation sites (Figure 4) suggest that spinal cord neurons are playing a role in the tempo-spatial integration of nociceptive information, leading to a reflex response which is distributed across multiple spinal cord segments and governed by an efficient defensive withdrawal.

Conflict(s) of Interest: None

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Ethical Permissions: Written informed consents were obtained from each volunteer previous to their participation. The local ethical committee approved this study (N-20180047).

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FIGURE LEGENDS

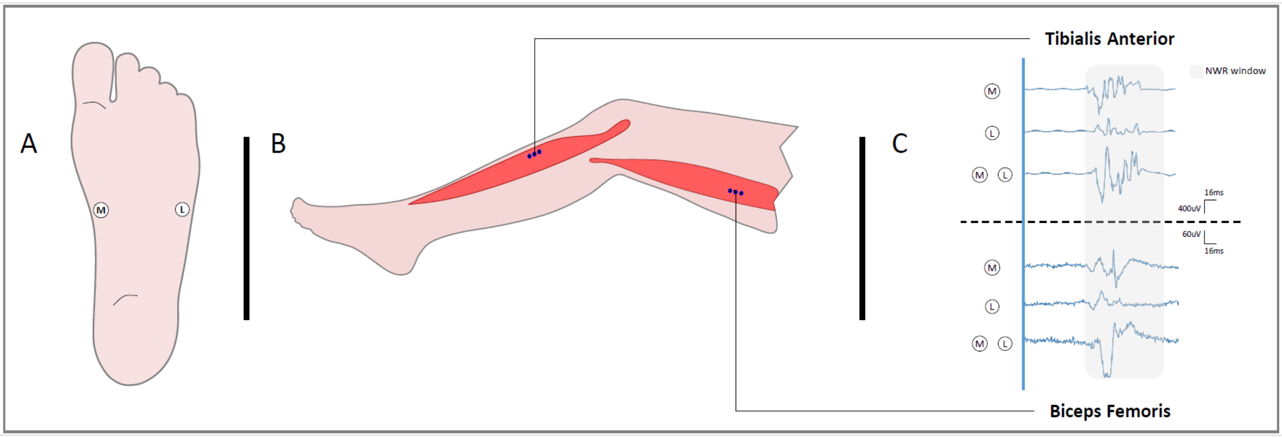

Figure 1: Diagram indicating the stimulated sites, recorded muscles and representative raw EMG traces obtained with Single and Simultaneous stimulation. A) Figure showing the location of the stimulating electrodes in the sole of the foot: M and L, medially and laterally located, respectively. B) Distal (Tibialis Anterior) and proximal (Biceps Femoris) muscles from which C) sEMG recordings were obtained. Raw EMG traces for both TA and BF illustrate the response of a representative subject to single and simultaneous stimuli in M and L sites.

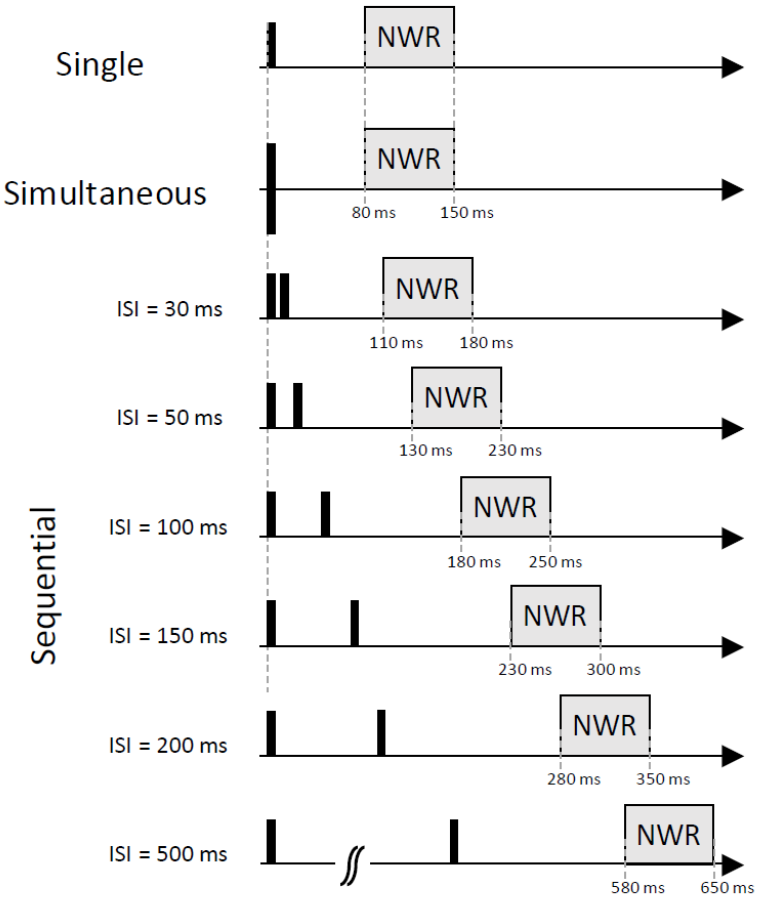

Figure 2: Stimulus types used in the experiment: Single, Simultaneous and Sequential. Black rectangles indicate stimulus artifact. The NWR was quantified in a 70 ms window (blue box) starting 80 ms after the trigger of second stimulation. Sequential stimulation was delivered with varying inter-stimulus intervals ranging from 30 ms to 500 ms.

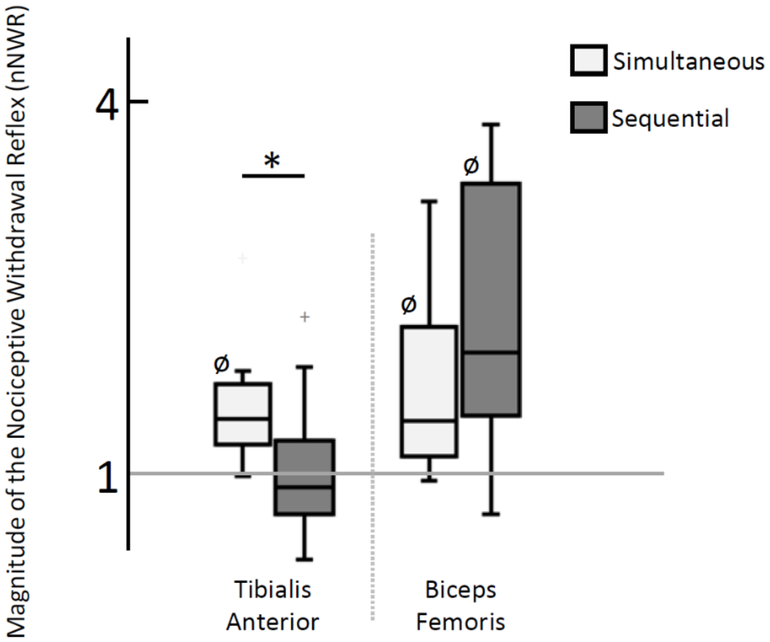

Figure 3: Box and whiskers plot showing nNWR for simultaneous and sequential (averaged across ISIs) stimulation in both TA (left) and BF (right) muscles. For TA muscle, there was a significant difference between simultaneous and sequential stimulation (*: $p<0.05$, Wilcoxon signed rank test). When compared to single stimulation (not shown), simultaneous stimulation showed increased reflex magnitude for both TA and BF muscles (ø: $p<0.01$, Friedman's test). Sequential stimulation, however, only provoked larger reflexes than single stimulation in BF (ø: $p<0.01$, Friedman's test).

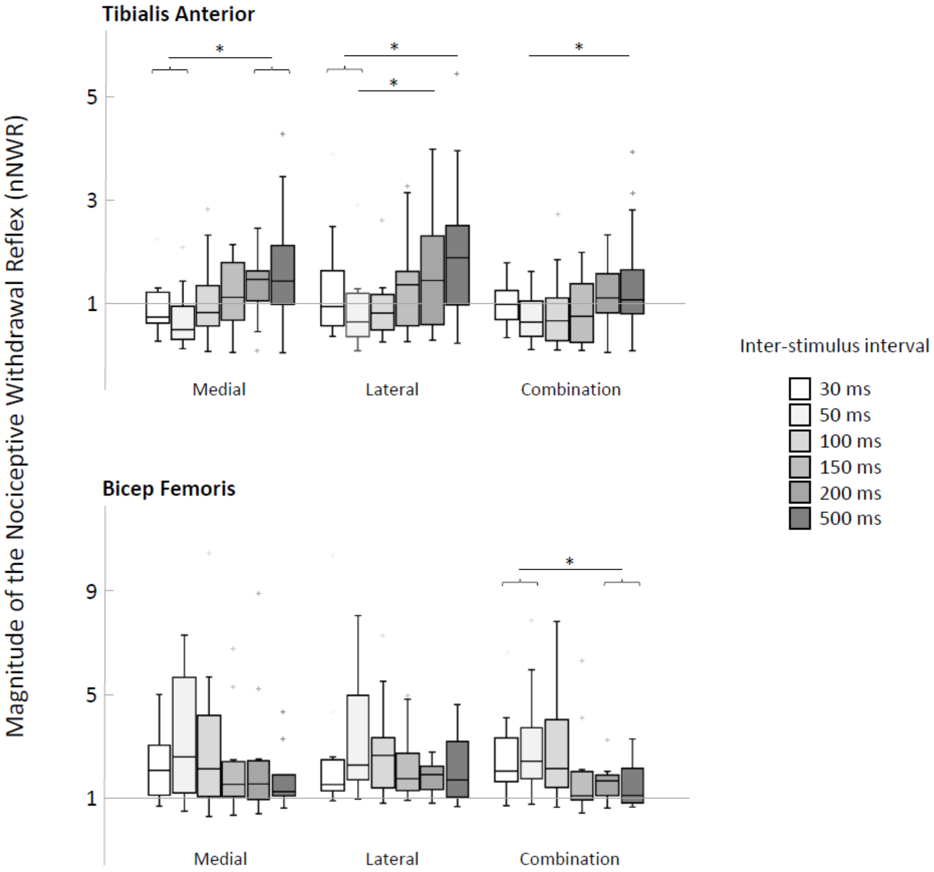

Figure 4: Box and whiskers plot illustrating the magnitude of the NWR when using sequential stimulation with different ISIs (30ms – 500ms). The top row shows the results obtained in the Tibialis Anterior muscle, while the bottom row displays the results of the Biceps Femoris muscle. ISIs. Values are shown as nNWR, normalized to the NWR due to single stimulation (see Methods section). Opposite tendencies in the proximal (BF, bottom row) compared to the distal (TA, top row) muscles were observed. For larger ISIs, TA-NWR was facilitated while BF-NWR was reduced (*: $p<0.05$, Wilcoxon signed rank test).

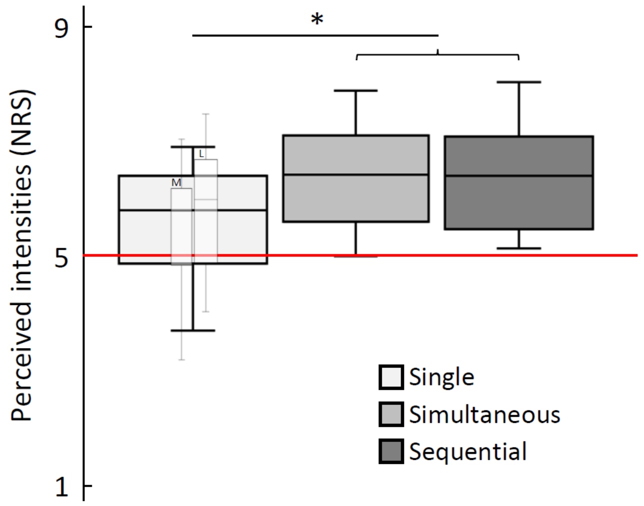

Figure 5: Box and whiskers plot showing Pain intensity ratings due to Single, Simultaneous and Sequential stimulation of M and L. Simultaneous and Sequential stimulation were perceived as more painful than Single stimulus (*p<0.05, Wilcoxon signed rank test). No significant difference was found between Simultaneous and Sequential stimulus. Pain threshold (NRS=5): red horizontal line.

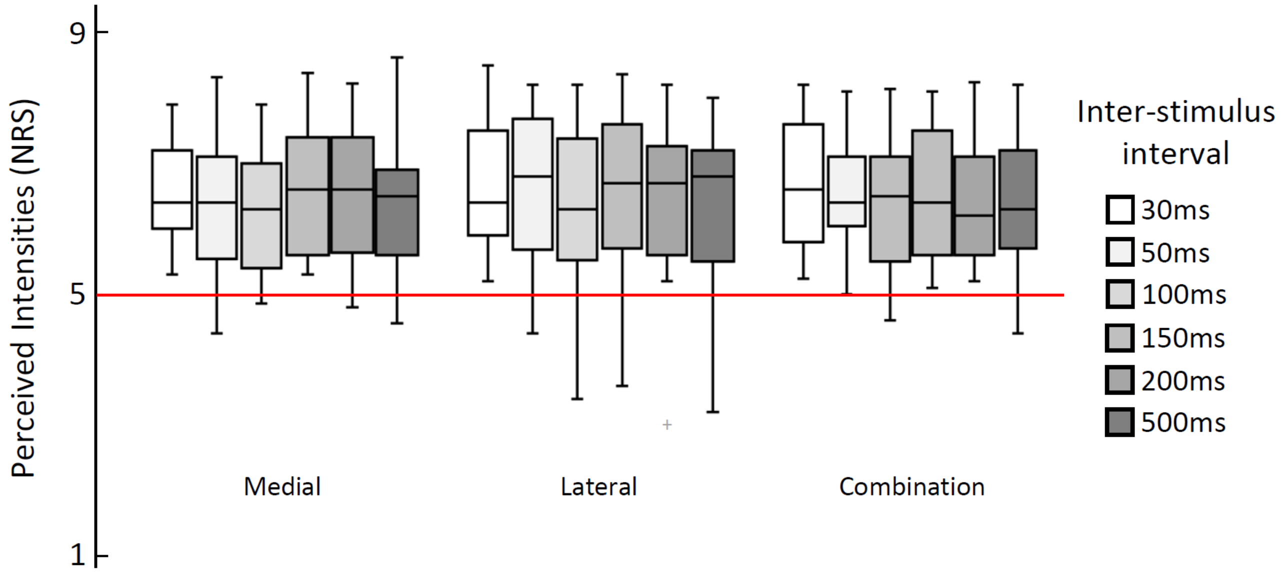

Figure 6: Box and whiskers plot of pain intensity ratings reported for Sequential stimulation in the medial electrode (M), in the lateral electrode (L) and as a combination of both (M-L). Different Inter-Stimulus Intervals (ISIs) are shown. Perception was above pain threshold (NRS=5: red horizontal line) in all cases, and there was no significant effect of ISI on the perception of pain intensity (p>0.05, Friedman's test).