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SPINAL TEMPORAL AND SPATIAL INTEGRATION OF MULTIPLE NOCICEPTIVE INPUT ASSESSED BY THE NOCICEPTIVE WITHDRAWAL REFLEX IN HUMANS

BY
MAURICIO CARLOS HENRICH

DISSERTATION SUBMITTED 2022

AALBORG UNIVERSITY
DENMARK

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CV

Mauricio Carlos Henrich was born in Buenos Aires, Argentina. He graduated as Bioengineer in 2016 from Universidad Nacional de Entre Ríos (Argentina). The research activities of his PhD project were conducted in the Center for Neuroplasticity and Pain (CNAP), Department of Health Science and Technology, Aalborg University. Mauricio's PhD project was supervised by Professor Ole Kæseler Andersen, and co-supervised by Associate Professor Ken Steffen Frahm. During the period between January 2020 and March 2020, research activities were performed at the Cincinnati Children's Hospital Medical Center (Cincinnati, Ohio, USA) as part of a collaboration with Professor Robert C. Coghill's group.

Mauricio's main areas of research are biomedical signal processing for the assessment of human nociception. In January 2021 he was appointed by Aalborg University as a Research Assistant in the Neurorehabilitation System group, Department of Health Science and Technology.

PREFACE

This PhD thesis provides a summary and discussion of the main findings of research conducted between 2018 and 2022 at the Center of Neuroplasticity and Pain, Aalborg University, Denmark. The three bellow studies disseminate the experimental work that was carried out during this project:

Study I [1]: M. C. Henrich, K. S. Frahm, and O. K. Andersen, "Spinal spatial integration of nociception and its functional role assessed via the nociceptive withdrawal reflex and psychophysical measures in healthy humans," Physiol. Rep., vol. 8, no. 22, pp. 11–20, Nov. 2020.

Study 2 [2]: M. C. Henrich, K. S. Frahm, and O. K. Andersen, "Tempo-spatial integration of nociceptive stimuli assessed via the nociceptive withdrawal reflex in healthy humans," J. Neurophysiol., vol. 20, no. 7, pp. 373–382, 2021.

Study 3 [3]: M. C. Henrich, K. S. Frahm, R. C. Coghill, and O. K. Andersen, "Spinal nociception is facilitated during cognitive distraction" - Interim Decision, BEING REVISED for publication in Neuroscience

The present document is organized into 4 chapters. Chapter 1 introduces the main concepts that will be discussed further on. It also states the general aim of the project and the research questions behind it. Chapter 2 discusses the evidence of spatial and temporal integration of nociception on animal and human studies. It focuses on spinal processing and discusses the main findings of Study I and II, in relation to relevant literature. That chapter deals with the research questions included in items 1, 2, and 3 (see section 1.5.1). Chapter 3 discusses on descending modulation of spinal nociception and how it can affect the spatial integration in the NWR pathway. It states and discusses the results obtained in Study III and aims at answering the last set of questions, item # 4. Chapter 4 discusses the main limitations of the methodologies used in the project. Lastly, Chapter 5 synthesizes the major findings of the project.

The articles and four conference abstracts disseminated the experimental work behind this project. The PhD project was supported by the Danish National Research Foundation (DNRF121), and by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 754465.

ENGLISH SUMMARY

The somatosensory system plays a predominant role in building an internal representation of the outer world together with the current state of the body. To this purpose, sensing organs continuously translate the physical properties of the environment into electrical signals that are conveyed to the brain to produce a cognitive perception of the surroundings. In addition to the cognitive response, humans have defensive mechanisms implemented within the central nervous system. These mechanisms allow rapid defense from potentially harmful stimuli to preserve homeostasis and avoid tissue damage. One example of such a defensive mechanism is the Nociceptive Withdrawal Reflex (NWR). The NWR is a polysynaptic spinal reflex that integrates information from sensory afferent fibers, proprioceptive fibers, together with descending modulatory activity, into an efficient withdrawal response of the exposed tissue. The optimal withdrawal response coordinates both lower limbs, the trunk, and potentially the entire body, to defend the exposed tissue while preserving balance, according to the current motor needs. To do this, temporal and spatial information from external stimuli needs to be integrated, encoded, and interpreted. Observations of spatial and temporal integration of nociception have been reported in the literature based on pain intensity ratings and other psychometrical variables (i.e.: location, radiation, quality). Whether and how the NWR pathway exploits tempo spatial information of the stimulus, and how that information becomes available to cognitive processes, remains to be clarified.

This thesis synthesizes the results of a PhD project that aimed at studying spinal spatial and temporal integration of nociception via the NWR. The project was motivated by recent evidence suggesting that lateral inhibitory mechanisms play a significant role in the spatial integration of multiple nociceptive stimuli applied in a small area of skin of healthy subjects. As early animal studies have shown spinal neurons encoding spatial characteristics of the stimulus, a spinal-specific approach was expected to provide relevant and novel evidence about the involved integrative mechanisms in humans. The primary outcome of all studies in this thesis was the magnitude of the NWR, which was complemented by other psychophysical outcomes. Additionally, the modulation of this spinal integration through descending control initiated by cognitive activity was investigated.

In particular, the first study on which this thesis is based was designed as a descriptive study that aimed at investigating spatial aspects of the integration of simultaneous nociceptive stimuli. Simultaneous stimulation included stimuli with varying inter-electrode distances (IEDs) applied through five electrodes on the sole of the foot. That study showed evidence of how spatial information of the stimuli is integrated into the NWR pathway. This integration seemed to have a functional, behavioral role according to the modular organization of the NWR. Evidence of spatial summation on both perceived intensity and NWR outcomes was presented and discussed. The

second study focused on how temporal information is integrated into an efficient withdrawal response. In this study, a temporal delay of different durations was used, together with single and simultaneous stimuli. The results provided evidence on how temporal and spatial aspects of the stimulus are integrated to produce a reflex and a perceptual response that is functional to the defensive role of the NWR. Differences between muscles involved in the NWR were studied and discussed. Lastly, the third study assessed whether a purely cognitive task modulates the integration of simultaneous stimulation at the spinal level. Two cognitive manipulations were used, aiming at shifting the attention of the subject away or into the stimulated site. Results showed that the NWR is significantly facilitated when subjects are distracted from the stimulation. The integration of simultaneous stimulation, however, seems not to be affected significantly.

In conclusion, the assessment of the NWR and its modulation by cognitive tasks provided novel evidence of the integration of nociception at the spinal level in healthy humans. The NWR pathway simultaneously integrates temporal and spatial information of the noxious stimuli, to elaborate an optimal defensive response. This net reflex response can be explained by a modular organization of the NWR with a functional role that likely involves the coordination of several muscles, according to the defensive needs. Results of the perceptual response (pain intensity) and the behavioral response (NWR) showed differential processing of information between the pain and the NWR pathways. This suggests that the NWR magnitude cannot be directly used as a proxy of pain intensity (particularly with complex stimuli) or spinal nociception.

DANSK RESUME

Det somatosensoriske nervesystem spiller en fremherskende rolle i opbygningen af en indre fortolkning af den ydre verden sammen med kroppens aktuelle tilstand. Til dette formål omsætter sanseorganer løbende omgivelsernes fysiske egenskaber til elektriske signaler, der overføres til hjernen for at producere en kognitiv opfattelse af omverdenen. Udover den kognitive respons har mennesker defensive mekanismer implementeret i centralnervesystemet. Disse mekanismer tillader hurtigt forsvar mod potentielt skadelige stimuli for at bevare homeostase og undgå vævsskade. Et eksempel på en sådan defensiv mekanisme er den nociceptive afværgerefleks (Eng: Nociceptive Withdrawal Reflex, NWR). NWR er en polysynaptisk rygmarvsrefleks, der inkorporerer information fra sensoriske afferente og proprioceptive nerver, samt descenderende modulatorisk aktivitet, for at opnå en effektiv tilbagetrækningsrespons af det eksponerede væv. Den optimale tilbagetrækningsrespons koordinerer både underekstremiteterne, torso og potentielt hele kroppen, for at forsvare det blottede væv og samtidig bevare balancen i overensstemmelse med de aktuelle motoriske behov. For at gøre dette skal temporal og spatiel information fra de ydre stimuli integreres, afkodes og fortolkes. Observationer af spatial og temporal integration af nociception er i litteraturen blevet rapporteret baseret på smerteintensitetsbedømmelser og andre psykometriske variabler (blandt andet placering, stråling, kvalitet). Hvorvidt og hvordan nervebanerne involveret i NWR bruger stimulussens temporal-spatielle information, og hvordan denne information bliver tilgængelig for kognitive processer, skal endnu afklares.

Denne afhandling syntetiserer resultaterne af et ph.d.-projekt, hvis formål var at studere spinal spatiel og temporal integration af nociception vha. NWR. Projektet var motiveret af nyere fund, der tyder på, at lateralt hæmmende mekanismer spiller en væsentlig rolle i den spatielle integration af flere nociceptive stimuli påført et lille hudområde hos raske forsøgspersoner. Da tidligere dyreforsøg har vist spinale neuroner, der afkoder for stimulussens spatielle karakteristika, forventedes en spinalspecifik tilgang at give relevant og ny evidens om de involverede integrerende mekanismer hos mennesker. Det primære resultat af alle undersøgelser i denne afhandling var størrelsen af NWR, som blev suppleret med andre psykofysiske resultater. Derudover blev moduleringen af denne spinale integration gennem aftagende kontrol initieret af kognitiv aktivitet undersøgt.

Især det første studie, som denne afhandling er baseret på, var designet som et deskriptivt studie, der havde til formål at undersøge spatielle aspekter af integrationen af simultane nociceptive stimuli. Simultan stimulering inkluderede stimuli med varierende interelektrodeafstande mellem fem elektroder placeret på fodsålen. Dette studie viste, hvordan stimulis spatielle information integreres i nervebanerne i NWR. Denne integration syntes at have en funktionel adfærdsmæssig rolle i henhold til NWR's modulære organisation. Beviser for spatiel summering af både opfattet

intensitet og NWR-resultater blev præsenteret og diskuteret. Det andet studie fokuserede på, hvordan temporal information integreres i en effektiv tilbagetrækningsrespons. I dette studie blev der brugt en temporal forsinkelse af forskellig varighed sammen med enkelte og simultane stimuli. Resultaterne gav belæg for, hvordan temporale og spatielle aspekter af stimuli er integreret for at frembringe en refleks og en perceptuel respons, der er funktionel i forhold til NWR's defensive rolle. Forskelle imellem muskler involveret i NWR blev undersøgt og diskuteret. Endelig vurderede det tredje studie, om en ren kognitiv opgave modulerer integration af simultan stimulering på rygmarvsniveau. To kognitive manipulationer blev brugt, med de formål enten at flytte forsøgspersonens opmærksomhed væk, - eller mod det stimulerede sted. Resultaterne viste, at NWR faciliteres, når individet bliver distraheret fra det stimulerede sted. Integrationen af simultan stimulering synes dog ikke at blive påvirket væsentligt.

Som konklusion gav vurderingen af NWR og dens modulering ved kognitive opgaver nye beviser for integrationen af nociception på spinalniveau hos raske mennesker. Nervebanerne i NWR integrerer samtidig temporal og spatiel information vedrørende smerte stimuli for at udarbejde en optimal defensiv reaktion. Denne samlede refleksrespons kan forklares ved en modulær organisation af NWR med en funktionel rolle, der sandsynligvis involverer koordinering af flere muskler relateret til de defensive behov. Resultaterne af den perceptuelle respons (smerteintensitet) og adfærdsresponsen (NWR) viste differentiel behandling af information mellem smerte- og NWR nervebaner. Dette tyder på, at magnituden af NWR ikke kan bruges direkte som en stedfortræder for smerteintensitet (især for komplekse stimuli) eller spinal nociception.

Tre artikler og fire konferenceabstrakter formidlede det eksperimentelle arbejde, der blev udført under dette projekt. Alle forsøgene blev udført på Center of Neuroplasticity and Pain, Aalborg Universitet, Danmark. Arbejdet blev støttet af Danmarks Grundforskningsfond (DNRF121) og af EU’s Horizon 2020 research and innovation programme under Marie Skłodowska-Curie-bevillingsaftale nr. 754465.

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This thesis is dedicated to my family, for their endless love and support:

To my grandmother Lali, who passed away while I was writing this thesis. Although she never understood why I had to emigrate to pursue my career, she always believed in me, certainly more than myself. I will always be thankful and in debt to her.

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LIST OF ABBREVIATIONS

IASP	International Association for the study of pain
DH	Dorsal horn
SC	Spinal cord
STT	Spinothalamic tract
WDR	Wide dynamic range
NS	Nociceptive specific
RF	Receptive field
SSP	Spatial summation of pain
TSP	Temporal summation of pain
SS	Spatial summation
LI	Lateral inhibition
NWR	Nociceptive withdrawal reflex
RRF	Reflex receptive field
FRA	Flexion reflex afferents
sEMG	Surface electromyography
IED	Inter-electrode distance
TA	Tibialis Anterior
PL	Peroneus Longus
ISI	Inter-stimulus interval
BF	Biceps Femoris
TDT	Temporal discrimination threshold
PAG	Periaqueductal gray
RVM	Rostroventral medulla
SDH	Superficial dorsal horn

CHAPTER 1. INTRODUCTION

1.1. PAIN

According to the International Association for the study of Pain (IASP), pain is defined as

> "An unpleasant sensory and emotional experience associated with, or resembling that associated with actual or potential tissue damage." [4]

The experience of acute pain can be understood as a defensive mechanism that signals (potential) tissue damage. Many pain-related pathologies, such as chronic pain, are characterized by pain that outlasts the normal healing time of acute tissue damage. Therefore, instead of being in phase with the aversive stimulation -if any-, the pain experience becomes chronic. Chronic pain has a significant negative impact on the quality of life of patients [5], [6]. It is estimated to affect between 12-30% of the general adult population [7]. Chronic lower back and neck pain was reported as the most frequent cause of disability, globally [5]. Additionally, it represents a major economic burden for the healthcare systems, being its estimated cost in Europe larger than €200 billion yearly [8], [9], with similar figures reported in the USA [10], [11]. How an acute process with a defined protective role evolves to a chronic pathological condition with apparently no useful purpose, remains unknown and highlights the need for further basic research in pain and nociception.

Although pain is normally described in terms of the mechanisms that signal nociception, it is important to differentiate these two concepts. The term nociception refers to the process of encoding the noxious characteristic of a stimulus and transmitting it through the nociceptive system [4], [12]. Peripheral nociceptive neurons transduce a noxious stimulus into a propagating signal that reaches the central nervous system. Via central neurons, this propagating signal potentially elicits behavioral reactions, such as defensive reflexes and autonomous responses. Eventually, this nociceptive signal can reach superior structures and produce the experience of pain. Nevertheless, it is important to highlight that the experience of pain is not necessarily correlated to the noxious stimulus, not in time, nor in intensity, spatial characteristics, or quality. Therefore, pain is a subjective percept that became available to the conscious experience under certain circumstances [4]. The pain system must encode, integrate, and interpret temporal and spatial aspects of the nociceptive stimulus if it is to elaborate a congruent pain experience and an associated relevant defensive response.

1.2. FROM A NOCICEPTIVE STIMULUS TO PAIN

Cutaneous nociceptors are activated by a noxious stimulus applied to the skin. They can be classified based on the nature of the stimulus optimally transduced in three major types: mechanical, thermal, and polymodal (this last group activated by intense mechanical, thermal, and chemical stimulation) [12]. Different types of fibers with anatomical and functional characteristics are generally associated with each type of nociceptor. Most mechanical and thermal nociceptors have small-diameter thinly-myelinated fibers with conduction velocities between 5-30 m/s (Aδ fibers) [13]. On the other hand, polymodal nociceptors have a small diameter, non-myelinated fibers with conduction velocities lower than 3 m/s (C fibers) [13]. A stimulus applied to the skin with sufficient intensity can trigger an action potential that conducts through a nociceptive fiber(s) to the dorsal horn (DH) of the spinal cord (SC). The DH constitutes the first synaptic stage of nociceptive neurons, from where they can project to superior structures [14]. Early electrophysiological studies in animals divided the gray matter of a SC section in ten different areas called laminas [15]. The DH compromises the first six layers of that division (i.e.: LI-LVI). Most nociceptive fibers directly synapse in the superficial layer (LI and LII) of the DH, however, all layers within the DH, directly or indirectly via interneurons, process nociceptive information [14]. Nociceptive information projecting from the DH to supraspinal centers does it so via five ascending pathways, each primarily targeting the thalamus (cervicothalamic and spinothalamic tracts), the reticular formation (spinoreticular tract), mesencephalic structures (spinomesencephalic tract), and the hypothalamus (spinohypothalamic tract). The main ascending nociceptive pathway is the spinothalamic tract (STT). Some STT neural bodies ascend from LI, although the majority of them are located in deeper laminae [16], [17]. A substantial proportion of STT neurons respond to a wide range of stimulus intensity, both noxious and innocuous (so-called wide dynamic range neurons, WDR), while a smaller proportion are nociceptive specific (NS). The majority of the second-order neurons in the superficial laminae are NS, while WDR are predominant in the deep dorsal horn (DDH) [14].

NS and WDR neurons integrate input from multiple neurons are likely to play a significant role in encoding sensory-discriminant and affective characteristics of the noxious stimulus such as its intensity, localization, and quality [18]–[22]. The specific role of each type of neuron is still under debate. However, some major differences between the activation profiles of NS and WDR neurons provide evidence on how they differentially encode the multidimensional aspects of pain and nociception. One of those differences is that WDR have large receptive fields (RFs) graded in sensitivity, and steeper stimulus-response curves [18], [20], [23]. The central zone normally responds to noxious and innocuous stimulus, and the sensitivity decrease towards the borders, where only noxious stimulation provokes a response. Additionally, the stimulus-response curve of rats DH-WDR neurons following repetitive noxious heat stimulation shows a similar trend as that of perceived pain

intensity in humans exposed to the same stimuli [18]. On the other hand, NS neurons have smaller RFs, an aspect that in early electrophysiological studies assigned NS neurons a primary role in localization of noxious stimulus [19]–[21]. Later evidence from animal studies in combination with imaging techniques in humans were in disagreement and suggested that WDR are primary encoders of pain intensity and other sensory aspects of the pain experience [18], [24], [25].

1.3. SPATIAL AND TEMPORAL INTEGRATION OF NOCICEPTION AND PAIN

Clinical observations emphasize the need for better understanding the mechanisms supporting nociception. In particular, patients suffering from pain disorders, such as chronic pain, phantom limb pain, fibromyalgia, complex regional pain syndrome, generally present with pain symptoms in widespread areas of the body that cannot be easily localized [26]–[29]. Abnormal spatial integrative mechanisms of nociceptive information are hypothetically playing an important role in the development of those symptoms [30]–[32]. Studies in humans with phantom limb pain [33], and complex regional pain syndrome [34] showed that sensory discrimination training can reduce pain intensity, and the decrease of pain is associated with improvements in task performance. These observations, although unable to prove a causal relationship, serve as an indication that a link between spatial integration of nociception and the development of pain symptoms likely exists and calls for further investigation.

Experimental evidence reflecting local spatial and temporal integrative mechanisms in humans has received considerable attention from the research community in the last decades [30], [35]–[38]. Particularly, observations that are commonly reported in the literature regarding nociceptive integration are spatial summation of pain (SSP), and temporal summation of pain (TSP). Spatial summation of pain (SSP) can be understood as the increase in perceived pain intensity (or decrease in pain threshold) when increasing the stimulated area. Early studies reported small or no effect of SSP when using heat stimulus on different skin areas [39], [40]. At odds with early reports are several more recent studies that repeatedly reported SSP in humans for thermal [31], [36], [37], [41]–[51], mechanical [32], [52]–[57], and electrical [58] stimulation. Although the exact mechanisms behind SSP are not completely understood, a combination of peripheral and central mechanisms likely explain how SSP is encoded by spinal circuits. A frequency coding on primary afferents might convey valuable information regarding the size of the stimulus (related to the size of the neuron's RF) at a peripheral level [20], [25], [59]. Centrally, local integration of many afferents on the same convergent neuron and the number of convergent cells recruited by the stimulus [25], [50], are likely playing a role.

Observations of SSP are generally sub-additive meaning that the increase in the perceived intensity is not proportional to the increase in the stimulated area. This observation raises the discussion of a potential inhibitory mechanism coexisting and interacting with spatial summation (SS). One of such local inhibiting mechanisms is Lateral Inhibition (LI). LI in neurobiological terms is defined as a phenomenon in which an excited neuron inhibits its surrounding neurons, via lateral inhibiting interconnections. The importance of the LI phenomenon in the somatosensory system was first established for the visual sense and then described for other senses [60]–[62]. By inhibiting activity in surrounding neurons, contrast is enhanced aiding discrimination and localization of somatosensory stimuli. On the nociceptive system, evidence of LI processes is scarce. However, a study by Quevedo and colleagues [35] has shown strong psychophysical indications supporting inhibitory effects between stimuli that are delivered in a small spatial distribution. Using laser stimulation in the form of a line, and a stimulus delivered as two discrete points separated a distance equal to the length of the line, pain intensity ratings were obtained and compared. Line stimulation provoked significantly lower pain intensity ratings than two-point stimulation, albeit stimulating a larger area and delivering more energy. A study by Marchand and collaborators [49] further showed that inhibitory mechanisms indeed exist and affect spatial integration of pain in healthy humans. In that study, progressive immersion of the entire arm in noxious hot water could not confirm SSP. Conversely, when the limb was initially fully submerged and then gradually withdrawn, SSP was confirmed. The authors then speculated that the gradual immersion of the limb in noxious hot water likely recruited both inhibitory and excitatory mechanisms simultaneously interacting to prevent significant SSP [49].

Temporal Summation of Pain (TSP) refers to the observation of increased pain ratings with repetitive noxious stimulation at a certain frequency [63], [64]. TSP is considered as the human counterpart of a very widely studied phenomenon of animal nociceptive processing, called wind-up [65]. The Wind-up phenomenon was originally defined as the progressive increase in the firing activity of central neurons due to repetitive activation of nociceptive primary afferents [65]–[68]. TSP/Wind-up in both humans and animals has been related to activation of the NMDA receptor at the spinal level [65], [69]–[71]. Its activation likely regulates sustained effects of a lasting neuroactive substance, released by primary nociceptive afferents [20], [72], [73] in the outer layers of the dorsal horn spinal cord [74]. TSP was reported for different types of stimuli applied in the body of healthy humans and patients [54], [75]–[82]. It is important to consider that the repetitive activation of nociceptive afferents and its transmission through the spinal cord is also subject to other inhibitory influences. These can have peripheral, spinal, and descending origins, and might be facilitated by the nociceptive input itself [83]. With the sequential stimulation of nociceptors, peripheral sensitization might also contribute to the increased perceived pain intensity observed in TSP experiments [31], [54].

From a nociceptive stimulus to the experience of pain, several stages of integration are present. As defined by the IASP, pain is highly subjective and depends on multiple factors that are not strictly related to the stimulus. Then, assessing spatial/temporal integration of nociception from a pain intensity outcome might not be representative of the integrative process. How temporal and spatial information is encoded and integrated through the human nociceptive system remains to be clarified. Evidence from animal studies suggests that temporal and spatial characteristics of a stimulus are mostly processed within the dorsal horn (DH) of the spinal cord, being the first convergent hub for afferent nociceptive information [20], [23], [84], [85]. Studies that directly assess neuronal activity in the spinal cord of animals, are, for obvious reasons, not possible to be performed in humans. Alternative indirect methodologies are of substantial importance to get valuable insight into human spinal nociception. One of those indirect methodologies is the quantification of a reflex response that is preserved in animals and humans: the so-called Nociceptive Withdrawal Reflex (NWR). Being a polysynaptic spinal reflex, the NWR has been considered as a potential proxy for spinal nociception and pain. This project was motivated by the general hypothesis that the indirect assessment of spinal nociception via the NWR will provide novel evidence about the processing of spatial and temporal information in the spinal cord of healthy humans. on human spinal nociception. Assessing these integrative mechanisms with a more objective tool focused on spinal processing might provide novel and valuable evidence, as elegantly stated by Bishop as early as in 1948:

> "Sensation is the apical fluorescence on the afferent tree of which the lower branches, at reflex levels, bear most of the fruit." [40]

1.4. THE NOCICEPTIVE WITHDRAWAL REFLEX TO ASSESS SPINAL NOCICEPTION.

The nociceptive withdrawal reflex (NWR) is a motor reaction elicited by a noxious stimulus applied to the body [86], [87]. It has a defensive role that consists of the removal of the exposed tissue from the potentially damaging stimulus. The neural pathway of the NWR includes primary afferent fibers, intraneuronal circuits within the DH of the spinal cord, the motor system, and descending modulatory commands from supraspinal structures [86], [87].

The first extensive description of a "flexor reflex" can be traced back to the first decade of the last century, when C. Sherrington described a characteristic reaction elicited by the noxious stimulation of the limb in cats and dogs [88]. As the major sign of this elicited reaction was a stereotyped flexion of the three major joints in the affected limbs, the author named the reflex the "nociceptive flexion reflex". Sherrington highlighted the defensive role of the reflex, based on the observation that the flexion of the affected limb was easily triggered with intense stimulation and

coordinated with a stepping response on the other limbs, named a “flight” response. The response that Sherrington observed was complemented by the extension of contralateral limbs in an attempt to preserve balance. That early study showed that the withdrawal reflex can be triggered by the noxious stimulation on different sites of the limb, although with a particular facility if stimulating the foot [88]. Although Sherrington noted that the NWR could be elicited from almost the entire limb, he recognized slight differences according to the stimulated site [88], [89]. By doing so, he proposed the term receptive field as an appropriate definition for the collection of sites from where a flexion reflex can be elicited when duly stimulated (later termed reflex receptive field, RRF) [88]. The fact that the referred neurophysiological studies were performed in decerebrated and spinalized animals suggests that the spinal pathway of the NWR is anatomically sufficient to maintain its basic defensive role. However, later animal studies showed that descending control tunes the NWR pathway to maintain its normal excitability levels and to preserve the defensive function of the reflex [90].

The concepts profiled by Sherrington’s work in animal studies were later translated into a new model: “Flexion Reflex Afferents (FRA)” [91]. The FRA model is a multisensorial reflex system that included afferent fibers from multiple receptors converging into the reflex pathway as a sensory feedback signal [92]. The convergence of this afferent information into a common set of interneurons within the spinal cord was understood as playing a modulatory role over motor behavior [92]. The nociceptive afferent information seems to be included independently of the rest of the pathway and treated by non-FRA pathways [92]. Although the FRA model served to explain the integration of afferent information with descending supraspinal control into a motor program, it fails to include nociceptive withdrawal patterns that are not solely characterized by flexion of major joints.

A new model was proposed in 1990 by J. Schouenborg and J. Kalliomäki that presented the NWR as organized in mostly independent pathways to different muscles [90]. They showed, via electrophysiological studies in rats, that a noxious stimulus applied in the skin triggers the contraction of only those muscles that produce the optimal withdrawal of the stimulated area. Then, the authors showed that the pattern of withdrawal (eversion/inversion, flexion/extension) largely depends on the site being stimulated [93], [94]. This spatial organization was further supported by a study in rats that showed a high correlation coefficient between withdrawal fields and receptive fields in several muscles of the hind limb [93]. The RRF of different muscles present some overlap between them, therefore a stimulus typically evokes the contraction of a group of synergistic muscles. Evidence of the link between the RRF and the withdrawal function was later reported in human studies [95], [96].

Evidence from studies in rats suggested that the encoding of the spatial characteristics of the RRF is implemented via intraneuronal circuits in the spinal cord [97]. The interneurons that encode the receptive field of specific muscles have a somatotopic

organized distribution in the DH, further supporting the modular organization of the NWR [97]. Worth noting is that such neurons cannot be antidromically driven from the cervical cord or the thalamus [97]. This suggests that the neural circuits putatively encoding the spatial organization of the RRF and the withdrawal pattern, are intrinsic spinal circuits.

Descending control over the NWR pathway is likely responsible for preserving its behavioral function. Studies in animals provided evidence of the latter by assessing changes in reflex excitability and RRF morphology after spinalization [94], [98]. The authors showed that after the lesion, reflex thresholds decreased and RRFs expanded. After the spinal shock, innocuous mechanical stimuli were sufficient to evoke reflex responses [94]. Additionally, the RRF of specific muscles expanded to skin areas that were not withdrawn with its contraction [94]. Similar evidence of abnormal reflex responses in humans with spinal disorders further supports that descending modulation is a key element to maintain proper defensive NWRs [99]–[101]. It is important to mention that the organization of the NWR pathway is not genetically inherited but established during development. This is supported by animal studies showing that neonatal spinalization results in an abnormal spatial input-output relationship between sensory input and withdrawal rection [102]. In addition, the development of that spinal organization seems to be driven by experience with the environment [103], since the adaptation of the reflex patterns is observed after neonatal transfer of tendons [104], and after changes of peripheral innervation [105].

The assessment of spinal nociception via the NWR both in man and animals [106] has been widely performed in clinical and research settings [86], [87] encouraged by early reports showing a high correlation between perceived pain and NWR outcomes [107], [108]. The NWR has the additional advantage that is an objective outcome to investigate spinal nociceptive mechanisms since it does not depend on cognitive self-reported ratings. More recent studies also confirmed the reliability of the NWR and RRF methodologies in healthy humans [109], [110] and in chronic pain patients [111]. Finally, as the NWR pathways are mostly contained within the spinal cord, speculations on the spinal role in nociceptive processing are possible and of interest.

1.5. AIM OF THE PROJECT

Pain is a multidimensional conscious experience. Its quantification via quality descriptors, intensity scales, localization tasks is a valuable approach aiming at disentangling aspects of that multidimensional experience. The main observations reported in the literature regarding spatial and temporal integration of pain are based on outcomes that are more related to the pain experience than to the nociceptive stimulus itself. Although this approach has definite value, it is argued that using a more spinal-specific methodology, novel evidence regarding spinal nociceptive

processing can be obtained. A large amount of evidence has suggested that spinal cord circuitry plays a significant role in the spatial and temporal integration of nociception. Therefore, it is proposed that the objective assessment of the NWR in healthy humans will provide novel evidence for the understanding of mechanisms behind the spatial and temporal integration of spinal nociception. Results are expected to provide new insight into the current understanding of mechanisms underlying pain, pain chronification, and pain modulation.

1.5.1. RESEARCH QUESTIONS ADDRESSED IN THE THESIS

As stated previously, spatial, and temporal integration of nociception are most likely playing a fundamental role in encoding multiple aspects of the pain experience. Evidence is somehow contradictory on how spatial and temporal integrative mechanisms affect pain perception and nociception. Animal studies have shown that DH neurons play an important role in processing spatial and temporal information of the noxious stimulus. However, direct assessment of central neurons is not possible in humans. By using the NWR as a proxy of spinal nociception, this project aimed at answering the following main questions:

1. Are simultaneous nociceptive stimuli integrated into the NWR pathway?
2. Is the NWR affected by spatial summation and/or lateral inhibition? Are perceived intensity ratings and the magnitude of the NWR similarly affected by spatial integrative processes?
3. How a temporal delay between simultaneous stimuli affects the spatial integration of the NWR? Is that integration differentially modulating proximal vs distal muscles?
4. Is it possible to engage descending control via a purely cognitive task to modulate how spinal circuits integrate simultaneous nociceptive stimuli? Is the NWR pathway modulated in the same direction as it is reported in the literature for pain intensity ratings?

Three studies were conducted trying to address the research questions, as follow:

***Study I:* "Spinal spatial integration of nociception and its functional role assessed via the nociceptive withdrawal reflex and psychophysical measures in healthy humans"** [1]

There is an apparent agreement in the literature that SSP is a key phenomenon behind encoding pain intensity. The NWR, at the same time, has a strong defensive behavioral value that would most likely benefit from an analogous spatial summation (SS) mechanism. Study I used single and double simultaneous electrical stimulation applied on the sole of the foot of healthy humans to elicit the NWR. The study aimed at investigating whether the magnitude of the NWR is indeed affected by SS (and/or LI), and whether the distance between stimulated sites modulates the sensory

integration in the reflex pathway. Besides NWR recordings, psychophysical outcomes were obtained to complement the assessment (see the full article for a detailed description of the methodology and results).

Study II: "Tempo-spatial integration of nociceptive stimuli assessed via the nociceptive withdrawal reflex in healthy humans" [2]

As introduced above and further discussed in the following chapters, SS seems to be a key mechanism in the encoding of pain intensity and the optimal NWR. This integration is most likely implemented within the spinal cord reflex pathway. Tempo-spatial integration of nociception is poorly understood. In Study II, we aimed at assessing whether introducing a temporal delay between simultaneous stimuli modulates the magnitude of the NWR. Different interstimulus intervals were used, and stimulation was delivered in the same or different sites of the sole of the foot. NWR was recorded and quantified in two different muscles. Psychophysical outcomes were also obtained to complement the assessment (see the full article).

Study III: "Spinal nociception is facilitated during cognitive distraction"[3]

Study III aimed at investigating whether descending control of the NWR pathway affects the integration of simultaneous stimuli applied on the sole of the foot. A purely cognitive paradigm based on attention/distraction tasks was used to engage descending control. The direction of the modulation was investigated in one proximal and one distal muscle. SS was expected to be modulated by the expansion/reduction of the RRF by the cognitive tasks (see the full article).

1.5.2. GRAPHICAL SUMMARY

A simplified diagram is provided in Figure 1 showing an overview of the relationship between the studies and the anatomical structures of interest.

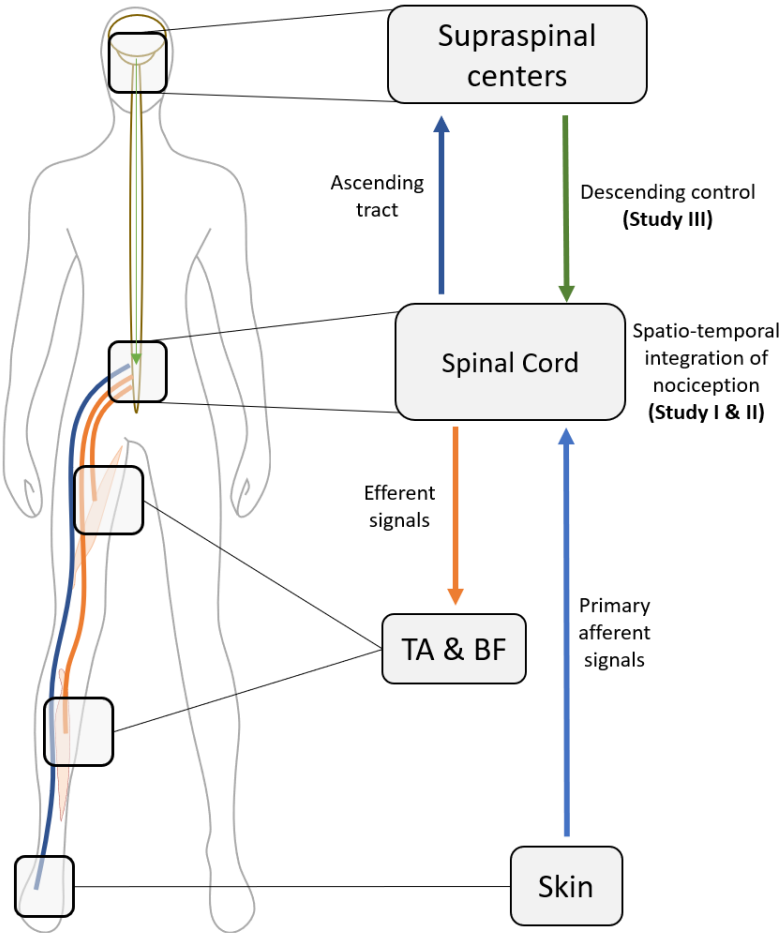

Figure 1: Schematic design illustrating different structures that are of interest to the project. Points of focus addressed in Study I, II, and III are identified in the diagram. Spatial and temporal integration of nociception at the spinal level was investigated in Study I and II. Study 3 focused on the descending modulation of such integration. All studies used the NWR as the primary outcome, quantified for Tibialis Anterior (TA) and/or Biceps Femoris (BF) muscles. The NWR was elicited by electrical stimulation of the skin applied on the sole of the foot of healthy subjects. In blue: primary afferent fibers and ascending tract. In orange: Efferent signals to Tibialis Anterior (TA) and Biceps Femoris (BF) muscles. In green: descending control from supraspinal structures.

CHAPTER 2. TEMPORAL AND SPATIAL INTEGRATION OF NOCICEPTION ASSESSED VIA THE NWR

2.1. OBJECTIVE ASSESSMENT OF SPINAL NOCICEPTION

As stated in the previous chapter, the NWR is a defensive reaction to protect the body from a potentially damaging stimulus. It is a behavioral response that is preserved in most animal species, such as the human, rat, mouse, cat, dog, rabbit, horse, pig, frog, among others [106], [112]. Since access to animal perception is not possible, nociception studies using animal subjects rely on the assessment of reflex responses as an objective way to interrogate the spinal processing of nociceptive information. Generally, the behavioral response is related to spinal cord neuronal activity by direct electrophysiological assessment of central neurons. Invasive recording is not possible in humans, therefore the quantification of the NWR via surface electromyography (sEMG) is a commonly used methodology that indirectly assesses spinal nociception in these subjects [86], [87], [113].

2.1.1. ELICITATION OF THE NWR

To elicit the NWR, a stimulus at suprathreshold intensity has to be applied to the body. The nature of the stimulus might vary, yet electrical stimulation is the most commonly reported in the literature [86], [87]. Natural stimulation, such as heat or pressure, has also been reported [88], [114]. However, they present methodological shortcomings that are avoided with electrical stimulation. The largest disadvantage of natural stimulation is that the timing between the stimulus onset and the recorded behavioral response is particularly difficult to control and measure reliably. Additionally, to avoid actual tissue damage, stimulation intensity has to be limited and not repeated over the same skin area. Electrical stimulation, on the other hand, is easy to deliver, and its intensity and onset/offset timing can be easily controlled and measured [115]. In all three studies of this project, electrical stimulation was used to elicit the NWR.

In the present project, electrical stimulation was delivered on the sole of the foot of healthy humans through small stimulating electrodes (see detailed methodology in published articles). The size of the stimulating electrodes was reduced to an area of 28 squared millimeters. Smaller electrode sizes were shown to produce sensations described as sharp, most likely indicating a larger proportion of Aδ fiber activation [116]–[118]. Electrical stimulation has the characteristics that it bypasses the receptor endings and directly depolarizes the innervating sensory fibers. Applied in the sole of the foot, as in the studies of the present project, it likely depolarizes thin fibers, although concurrent activation of other fibers cannot be completely discarded[118].

In Study I, II, and III, the intensity of the electrical stimulus used to elicit the NWR was set based on an estimation of NWR-threshold (NWR-th). A standardized criterion outlined by a series of studies made by Rhudy and colleagues [119] was used to automatically detect the presence of a NWR. That criterion is based on the interval peak z-score exceeding a threshold value of 12 [119]–[121]. The criterion was implemented in an automated staircase protocol that determined the threshold values [120].

2.1.2. QUANTIFICATION OF THE NWR

Recording of muscular activity involved in the NWR can be done via intra-muscular or by sEMG. Intramuscular recordings have the advantage that they are robust to contamination from activity in nearby muscles (crosstalk) [122]. However, it presents a serious disadvantage being an invasive recording. In addition to the discomfort induced in the participant, the recordings might be biased by the pain/discomfort produced by the recording technique itself.

sEMG to register the NWR is generally performed by using two recording electrodes mounted on the skin over the muscle of interest [86], [87], [123]. In the present project, studies were performed using double differential sEMG recordings, since it was previously demonstrated to maintain sensitivity and improve specificity when assessing the activity of muscles in the lower limb [124]–[126]. Several methods for the estimation of the magnitude of the reflex response are available and reported in the literature, e.g.: root mean square values, areas under rectified curves, peak to peak amplitudes, mean amplitudes, among others. In Study I, II and III, the magnitude of the NWR was quantified by calculating the root-mean-square value of the recorded signal in a predefined reflex window (compatible with Aδ-fiber conduction velocities), as it is commonly reported in the literature [86], [87], [95], [127]–[142].

2.2. SPINAL SPATIAL INTEGRATION OF NOCICEPTION

Electrophysiological studies in humans and animals provide evidence of the neural substrate behind the spatial integration of nociception at the spinal level. Price and collaborators [50] showed early evidence on a set of mechanisms that are likely to support SSP. Frequency encoding by primary afferent fibers is likely an early integrative mechanism playing a role in SSP at the peripheral level [25]. The net output (discharge frequency) of a certain afferent neuron depends on the portion of its RF that is stimulated (local integration [50]). As discussed by Price and collaborators [50], another mechanism that is likely involved in SSP is the gradual recruitment of a population of neurons when the stimulated area increases. Neuronal recruitment likely plays a role when the simultaneous stimuli are separated by such a distance that convergent neurons' RF do not overlap significantly. Then, the number of second and

third-order neurons recruited will increase, potentially enhancing the afferent inflow that reaches superior structures [50].

The neural mechanisms behind spatial summation in the NWR pathway might likely be explained by similar integrative phenomena as in SSP. Direct assessment of neuronal activity in central neurons of animals provides valuable insight into how spatial information can be encoded in the nociceptive system. Studies in rats by Schouenborg and collaborators [93], [97] demonstrated that DDH neurons, most likely WDR-type neurons, encode the spatial input-output relationship of the nociceptive withdrawal reflex. The authors showed that the aforementioned neurons cannot be activated antidromically from the upper cervical cord, suggesting that those cells were interneurons in the DDH [97]. The convergence of multiple nociceptive afferents into those interneurons is likely an important mechanism playing a role in spinal spatial integration. An increase in discharge frequency of spinal WDR neurons might be a mechanism by which the afferent inflow caused by multiple nociceptive stimuli summate spatially in the spinal cord [143].

Data from studies using a single stimulus of varying intensity provide indirect evidence of SS since increasing stimulus intensity activates a larger number of peripheral nociceptive fibers. Of relevance for this project is a study by Coghill and collaborators in animals [24]. Using image analysis, the authors showed increased metabolic rate (glucose utilization) on spinal neurons with the increase in stimulation intensity [24]. Laminae V-VII presented particularly intense activity and rostrocaudal population recruitment, suggesting that deep WDR neurons play an important role in encoding summation in spinal nociceptive processing [24].

Human spinal nociception can be investigated indirectly via the quantification of the NWR. Generally, human studies involving the NWR use a single stimulus to probe spinal nociception [86], [87]. Exceptions are those that use both conditioning and a test stimulus to assess remote inhibition or conditioning pain modulation [144]–[146]. However, other mechanisms of integration and pain modulation are involved and make it difficult to disentangle partial effects. Evidence of spatial summation assessed via the NWR in healthy humans is scarce. Study I [1]showed that paired stimulation elicits significantly larger NWR than single stimulation. The magnitude of the NWR increased 73% when the area of stimulation doubled in size, suggesting SS in the NWR pathway. The spatial summation of the NWR seemed to be subadditive, similarly as with SSP (see below).

As discussed before, SS due to neuronal recruitment might be optimally facilitated separating the paired stimuli, since the probability of stimulating different RFs increases. In Study I [1], different IEDs were used ("IED 1"=1.5cm, "IED 2"=3cm, "IED 3"=4.5cm, "IED 4"=6cm) to assess the effect of distance on the spatial integration. A significant effect of distance was found, and post hoc comparison confirmed that smaller IEDs induce significantly larger NWR. By increasing the IED

from 1.5cm to 6cm, the magnitude of the NWR decreased from being 91% to 54% larger than that elicited by single stimulation. The subadditive nature of the summation might be indicating that, when simultaneously stimulating different RRFs, the balance between facilitating and inhibiting processes shifts toward inhibition with increasing IED. The inhibition of the TA-NWR observed when simultaneously stimulating the medial and lateral side of the sole of the foot might be explained by a mechanism of inhibition between adjacent RRFs. As introduced before, the stimulation within the RRF of a certain muscle or group of synergistic muscles elicits a NWR by contracting those specific muscles. Contrary, the stimulation outside its RRF likely fails to produce contraction and it can also inhibit the reflex. Evidence from human [140] and animal [97], [147] studies suggest that mechanisms of inhibition between RRF indeed exist. In Study I [1], a schematic model of inhibition between lateral RRF is presented that might explain the observed results.

Animal studies confirmed that spinal neuronal networks indeed encode spatial characteristics of the stimulus to elaborate a reflex response [148]. Similarly, the results of Study I [1] seem to indicate that spinal nociceptive processing integrates spatial information of the stimulus to elaborate the optimal reflex response in healthy humans. Whether that information is preserved through the whole integrative process, and whether it becomes available to the human subjective experience of pain, remains elusive to clarify. Particularly since, as introduced before, from a nociceptive stimulus to the experience of pain, information is filtered and integrated several times before reaching the cortex (for the STT: SC, thalamus, cortex). Results of Study I showed that the integration of simultaneous stimuli in the NWR pathway differs from that of perceived intensities (see below).

2.3. SPATIAL INTEGRATION ON PERCEIVED INTENSITIES

SSP was repeatedly observed in human studies for stimuli of different nature and applied in diverse sites of the body. SSP is usually reported as either a reduction of pain threshold or an increment in perceived pain intensity when the stimulated area increases. SSP significantly affects the perception of pain intensity, and it is an important phenomenon in the codification of spatial characteristics of the pain experience [41], [45], [50].

The results of Study I [1] agree with the literature regarding SSP, since paired stimulation caused significantly larger perceived intensities than single stimulus. SSP was sub-additive in Study I [1], a 100% increase in the area of stimulation produced an approximately 30% increase in perceived intensity. Studies reporting SSP when increasing the stimulated area, are abundant in the literature [25], [31], [32], [36]–[38], [41]–[45], [48]–[50], [52], [54], [57], [58], [149]–[155], and all agree in the sub-additive characteristic of the integration.

Human studies assessing distance-based spatial summation of pain showed that by increasing the IED, larger pain intensity ratings are produced, most likely due to population recruitment [50], [58], [156]. In Study I [1] different inter-electrode distances (IEDs) were used to assess spatial integration of nociception based on the distance between stimuli. The results showed a significant effect of IED suggesting the presence of distance-based SSP, even though corrected multiple comparisons failed to identify which pair of IED were indeed statistically different. Other studies that assessed distance-based SSP reported similar findings. Of particular relevance are those of Reid et al. [58] and Defrin et al. [44], [150], [156]. In the study of Reid and collaborators, electrical stimulation (similar to the one used in Study I but applied in the forearm) showed SSP that monotonically increased for IED of 0cm, 5cm, 10cm, and 20cm. The results of Defrin and collaborators also agreed that SSP seems to be relatively stable for distances between 0cm to 25cm [156]/30cm [44], and to decrease thereafter. It is important to note that the distances used in Study I ranged between 1.5 cm and 6 cm. Larger IEDs could not be tested since stimuli were applied in the sole of the foot to elicit the NWR. Considering that the IEDs used in Study I [1] (IED=1 equivalent to 1.5 cm; IED=2 to 3 cm; IED=3 to 4.5 cm and IED=5 being 6 cm) tend to induce a monotonic increase in perceived intensities, it seems that results are comparable with the available literature and might be explained by the described mechanisms behind SSP.

2.4. DIFFERENTIAL INTEGRATION BETWEEN PERCEIVED INTENSITIES AND THE NWR

As discussed above, Study I showed novel evidence of spinal integration of nociception, assessed via the NWR in healthy humans. Sub additive spatial summation was found on the NWR pathway. When separating the paired stimuli, the magnitude of the elicited NWR was reduced, suggesting the presence of facilitated inhibition with larger IEDs. The inhibition of the NWR in the Tibialis Anterior (TA) when stimulating with IED 3 and 4, might be explained by the modular organization model of the NWR. As stated in the previous chapter, this organization is most likely implemented in the DDH of the SC [97], [157], and might be similar to the one in humans [87], [95], [96], [129]. In Study I, conditions of IED 3 and IED 4 are simultaneously stimulating the arch and the lateral side of the sole of the foot [1]. As proposed in the model of Schouenborg and Kalliomaki [90], and confirmed by other studies in animals [97], [147], [148] and humans [95], [96], [99], [129], [140], [158], the recruitment of muscles depends on the site being stimulated. The functional role consists in optimally withdrawing the skin area that is exposed to the stimulus. All those studies mentioned above were implemented with single stimulation in different sites. According to that evidence, the single stimulation in the arch of the foot would elicit the inversion and flexion of the foot, optimally produced by the contraction of the TA muscle [96], [127], [129], [158]. Contrary, the stimulation of the lateral side

of the sole of the foot most likely elicits eversion of the foot, which is optimally served by activity in the Peroneus Longus (PL) muscle [96]. The optimal withdrawal strategy for the simultaneous stimulation of both the medial and lateral side of the sole of the foot (as in conditions IED 1 and 4 of Study I), will no longer be served by the contraction of the TA or PL. More complex recruitment of muscles that stabilize the talocalcaneal joint might be the optimal response from a defensive and functional perspective. The reduction in the magnitude of the TA NWR observed in Study I for larger IEDs might be explained by this functional organization.

Contrary, perceived intensities seem to be facilitated with increasing IEDs. This integration is likely responding to the defensive role of the pain system, since simultaneous stimulation of distant areas (<30cm) might indicate a threat with a larger potential to harm the body. Separating the paired stimuli likely increases the number of WDR neurons that are activated in the DDH of the SC. Therefore, the net output projecting to superior structures increases producing an enhanced perceived intensity.

2.5. NOCICEPTIVE PROCESSING OF TEMPORAL CHARACTERISTICS

The modular organization of the NWR provides an explanation for the site effect on the NWR patterns. Those patterns are implemented by differential recruitment of muscles that optimally serve the defensive withdrawal. The RRF of specific muscles can be determined as the skin area from where to elicit the contraction of a specific muscle (or group of synergetic muscles). The spatial characteristics of the RRF are likely determined by DDH neurons, as inferred from studies in animals [97], [157]. Those neurons likely integrate and encode spatial characteristics of a noxious stimulus. Indeed, the results of Study I [1] showed indirect evidence and discussed the potential role of this neuronal circuit on the SS of nociception in the NWR pathway. Particularly, Study I [1] suggested that simultaneous stimulation of different sites of the sole of the foot is integrated by spinal neurons. Study II [2] investigated whether the introduction of a temporal delay (inter-stimulus intervals, ISIs) between stimuli delivered in different or the same site(s) affects the integration of nociception at the spinal level. That study hypothesized that short ISIs are integrated at spinal levels and facilitate NWRs. How the integration affects different muscles is unknown and was assessed in Study II by recording the NWR in TA and Biceps Femoris (BF) muscles. Perceived pain intensities were obtained as secondary outcomes. Finally, temporal discrimination of nociceptive stimuli was assessed when stimulating on the same or different sites to investigate whether temporal integration incorporates spatial information on the perception.

Direct recording of extracellular activity in the spinal cord of cats showed that the repetitive stimulation of primary C fibers is integrated by dorsal horn spinal cord

neurons inducing facilitation of spinal cord neuronal activity [67], [68], [159]. Further evidence suggested that the repetitive stimulation of Aδ fibers could also induce similar facilitation [159], [160]. This phenomenon is called wind-up and it is believed to play a role in initiating central sensitization [159]. Windup is an observation reported exclusively in animal studies since the direct recording of central neurons cannot be performed in humans. TS-NWR can be considered the human counterpart of the windup phenomenon. The NWR pathway integrates temporal information of the nociceptive stimulus when these are delivered on the same site. This can be observed as a facilitation of the NWR by delivering repetitive stimulation, a phenomenon called temporal summation of the NWR (TS-NWR) [75], [161]. Results of Study II showed that sequential stimulation (averaged between ISIs) abolished SS in TA, while the magnitude of the BF-NWR was still significantly facilitated. A priori, these results indicate that proximal and distal muscles might be differentially modulated according to temporal characteristics of the nociceptive stimulus. When assessing the effect of the different ISIs, opposed tendencies were confirmed between TA- and BF-NWR. For TA, short ISIs (30ms and 50ms) provoked smaller NWR than longer ISIs (250ms and 500ms). The opposite tendency was observed for BF. Interestingly, these tendencies seemed to be independent of the stimulated site(s). Stimulation frequency dependence of TS-NWR was previously shown in human studies [75], [161]–[164] as an increment on the magnitude of the NWR with increasing stimulation frequency. Those studies quantified the NWR by sEMG only on proximal muscles. Results of Study II regarding BF-NWR agreed with those and might be explained by a process of temporal summation with repeated stimulation at smaller ISIs (higher stimulus frequencies) [159]. On the other hand, the results of the TA-NWR showed the opposite behavior, a differential effect of ISI (or stimulus frequency) on distal (TA) and proximal (BF) muscles, suggesting that the degree of temporal summation might differ between muscles.

Based on the modular organization of the NWR, it is possible to speculate that the divergent effect of ISI for TA and BF serves a defensive role. Shorter ISIs are more likely to be integrated as a single nociceptive event (see also results on temporal discrimination below). Therefore, particularly for ISIs of 30ms and 50ms, if the nociceptive system is not capable of discriminating the two stimuli, they might be interpreted as a single but longer stimulus with more potential to produce damage. To protect the body from harm, the optimal defensive reaction would be to withdraw the entire limb from the stimulus. Due to the anatomical location of the BF muscle, its contraction serves the flexion of the knee and hip. Therefore, it produces withdrawal of the entire lower limb, rather than a tuned site-dependent response [96], [140]. Facilitating the recruitment of proximal muscles, such as the BF in Study II, might serve to that optimal defensive response, and might explain those observations of Study II.

Analogous to TS-NWR, TSP is reported as increasing pain intensity ratings with repetitive nociceptive stimulation [20], [155]. Interestingly, Study II failed to

demonstrate an effect of ISI on perceived intensity ratings. Other studies have shown significant increases in pain ratings starting at the third repetition or later [77], [161]. In the light of that evidence, it seems that the NWR pathway is more sensitive to repetitive stimulation since a larger number of stimuli are likely needed to induce TSP than TS-NWR. Mean values of temporal discrimination thresholds (TDT) were 84.1ms for stimulation on the medial side of the sole of the foot, 95.5ms on the lateral side, and 71.0ms when combining both sites (medial-lateral). Smaller TDT for the latter stimulus seem to suggest that temporal discrimination exploits spatial information of the stimulus, and that the discrimination is enhanced when stimuli are delivered in different sites.

Finally, simultaneous stimulation of both sites elicited significantly larger NWRs and perceived intensities than single stimulation, confirming SS on the NWR for both TA and BF muscles, and SSP. SS was subadditive for both outcomes, confirming similar results as in Study I [1].

CHAPTER 3. DESCENDING CONTROL OF SPINAL NOCICEPTION

As acknowledged by the IASP (see Introduction), pain is a highly subjective and multidimensional experience [4]. Somatosensory processing of innocuous stimuli permits the sensing of the environment and of the current state of the body. The arousing experience allows perception of the external world. Contrary, the object of the pain system is the body itself integrated with the external world [165]. This can be exemplified by the simple experiment of touching a warm vs a hot stove. The warm sensation perceived in the first case would be attributed to the stove, one normally says "the stove is warm". However, if one touches a stove hot enough to produce pain, the pain perception is attributed to the part of the body that touched the hot stove -and one might normally express "my finger hurts". Although this difference might seem trivial, it represents that the perception of pain is not linearly related to the external stimulus. Moreover, it seems to be more related to interoceptive processing and less to the external object that provokes the sensation.

Assuming that pain is highly subjective, and heavily related to internal processing, one might ask the question of whether the experience of pain can be endogenously modulated. An early study made by Beecher H. [166], involving humans suffering from war wounds seems to provide evidence of this endogenous modulation of pain. In that study, the author compared the significance of wounds with the pain experienced in two different groups of people: civilians and war soldiers after being taken from the battlefield to the field hospital. The study showed no clear relationship between the extent of the wound and the perception of pain. It concluded that the experience of pain was dependent on what the wound signifies to the person, suggesting that individual levels of anxiety were likely playing a significant role. Additional examples of endogenous modulation of pain are nowadays present in the literature. In addition to anxiety [167]–[170], emotions [168], [171]–[178], and other cognitive factors [133], [179]–[182] were shown to modulate nociception and pain.

The anatomical and physiological basis behind the modulation of spinal nociception via descending control is fairly well documented. Studies in animals showed already in the late 60s that electrical stimulation of certain brain structures produces pain behavior analgesia [183]. The brain structures that most effectively induced analgesia were in the periaqueductal gray matter (PAG) [183]–[185]. Those studies in rats and cats were complemented by reports of electrically induced analgesia in human subjects [186]–[188]. Those early findings suggesting the presence of an effective endogenous analgesia system motivated the study of the most thoroughly described descending analgesic pathway in humans, the periaqueductal gray matter-rostroventral medulla (PAG-RVM) system.

Neurons in the PAG project to the RVM from where the modulation is exerted into the SC. Although initially considered as a purely antinociceptive system, more recent studies demonstrated the biphasic nature of the modulation that the PAG-RVM system can produce [189]–[192]. Fields and colleagues in 1983 reported the presence of two populations of neurons (off- and on-cells) within the RVM, that had an opposite firing pattern associated with a nociceptive reflex in rats [192]. Later, many studies confirmed that those off- and on- cells originally proposed by Fields and colleagues were the inhibitory and facilitatory output from the PAG-RVM system, respectively [193]. The DH of the SC is the first synaptic target of primary nociceptive fibers, and also an ideal target for the PAG-RVM system to modulate nociception. Due to those inhibitory and facilitatory neural populations in the RVM, biphasic modulation of spinal nociception descending from that structure is possible [193].

3.1. COGNITIVE TASKS TO INDUCE TOP-DOWN MODULATION

In Study III, it was investigated whether it is possible to engage descending control of nociception by a cognitive manipulation to affect spinal integration of simultaneous stimulation. Selecting a cognitive manipulation to induce modulation towards two directions (pro/anti-nociception) is not trivial. Some studies have already shown that emotions with negative/positive valence might be an efficient means to engage descending control inducing anti/pro-nociceptive states [174], [194]. One cognitive manipulation that has been demonstrated to affect pain perception is attentional shifts [195], [196]. In this regard, it is relevant to highlight the results obtained by Quevedo and collaborators [153]. The authors of the latter study instructed healthy humans to assess pain intensity due to paired noxious stimulation under three different attentional tasks: giving an overall rating for the paired stimuli, individual ratings for each stimulus, or only for one of them. While rating both stimuli together induced significant SSP, dividing the attention of the subjects suppressed SSP. They demonstrated that attention shifts dynamically modulate the integration of multiple stimuli in the perception of pain intensity.

Various supraspinal structures are likely involved in attentional processes modulating pain perception, such as the Cingulate Cortex, Prefrontal Cortex, Superior Parietal Cortex, Thalamus, Insula, Amygdala, and the PAG [195], [196]. A diffusor tensor imaging study in human subjects [197] provided anatomical evidence for the link between the PAG and some of those rostral structures likely involved in cognitive modulation of descending control (Prefrontal Cortex, Amygdala, Thalamus). In Study III, a modified version of the Stroop test [198] was used to shift the attention of the participants away from the stimulus (see the full article for detailed methodology). Distracting tasks as the one used in Study 3 have been shown to decrease pain intensity perception associated with increased activation of Prefrontal Cortex [199], Cingulate Cortex [199], [200], Thalamus [200], and PAG [200], [201] (see Figure 2).

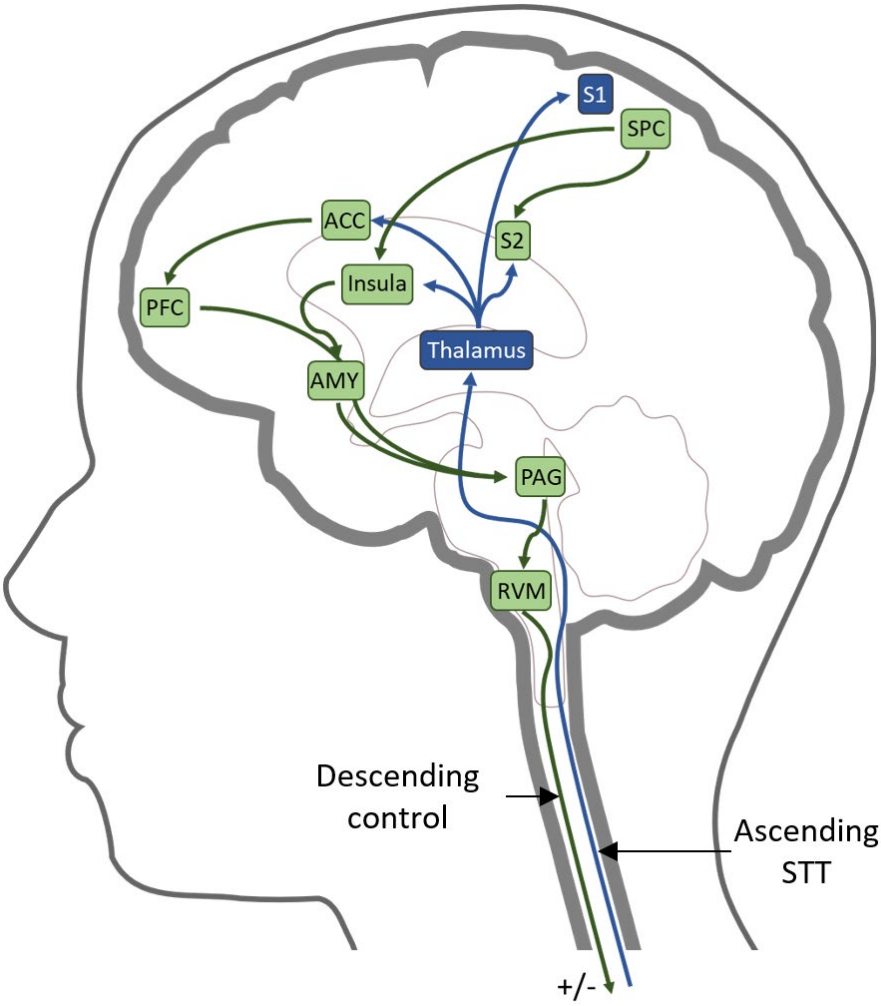

Figure 2: Illustrating scheme showing main supraspinal structures likely involved in pain and its descending control by cognitive/emotional tasks [195]. *The ascending spinothalamic tract (STT) is the main afferent pain pathway entering the brain from the SC (in blue). The figure also depicts the main thalamic projections: Anterior Cingulate Cortex (ACC), Insula, Primary (S1), and Secondary (S2) Somatosensory Cortex. Supraspinal structures likely involved in the descending modulation of nociception by cognitive tasks are depicted in green. The net descending control by the PAG ultimately relayed by the RVM might be excitatory or inhibitory. PFC: Prefrontal cortex; ACC: Anterior cingulate cortex; AMY: Amygdala; SPC: Superior Parietal Cortex. See the text for discussion and references.*

Study III focused on cognitive tasks with the potential to engage top-down modulation in both directions over the NWR pathway. Of particular relevance for this project are

the results of Bjerre and colleagues [182] in healthy humans since they focused on reflex responses instead of pain perception outcomes. In that study, it was shown that manipulating the attention of the participant away (distraction) or towards (attention) the stimulated area, effectively shaped the RRF of the TA muscle. In particular, distracting the subject from the stimulus induced a pronociceptive-like state, in which the TA-RRF was significantly expanded (from covering the arch of the foot to almost the entire sole of the foot). Contrary, attending to the stimulation significantly reduced the TA-RRF. The expansion and reduction of the TA-RRF by a cognitive task were understood by the authors as a dynamic descending control affecting spinal spatial integration of nociceptive stimuli [182].

The hypothesis of Study III assumes that attentional drives can shape the RRF of WDR neurons in the dorsal horn of the spinal cord and propose that this modulation can affect the integration of paired stimuli. Specifically, in Study III it was hypothesized that the distraction task will enlarge the RRF of TA to include the lateral side of the sole of the foot. Then, the simultaneous stimulation of both the arch and the lateral side of the sole of the foot will be integrated producing a larger NWR than in baseline conditions. A condition of attention was included to assess whether a contraction of the RRF can be induced to prevent paired stimuli to be integrated.

Early animal studies provide critical evidence supporting the hypothesis of Study III, regarding the link between the shape of the RRF and the spatial integration of nociceptive stimuli applied within it. Particularly relevant is a study in rats showing that receptive fields of WDR neurons located in the DDH of the SC are highly correlated with RRF of specific muscles [97]. These groups of neurons are organized in pools according to the related muscle within the DDH. These WDR neurons, considered as putative reflex encoders are likely responsible for the spatial integration of nociceptive afferent information, as discussed in Chapter 2.

Results of Study III [3] showed that the distraction task induced net facilitation of the NWR in both TA and BF. The NWR during distraction was significantly larger than during baseline for all stimulated sites, with no significant difference between stimulation sites. The summation observed for paired stimulation was not statistically different between baseline and distraction conditions. Therefore, although a net facilitation of the NWR seemed to be induced by the distraction task, the expansion of the TA/BF-RRF, if present, did not seem to affect the spatial integration of paired stimuli. This might be suggesting that spatial integration is robust to descending control, at least in the experimental conditions of Study III. The observation of the expansion of the RRF made by Bjerre and colleagues [182] might be the result of a net increase in the gain of the spinal nociceptive system induced by the distraction task, as seem to be the case in Study III. The distraction task might likely have engaged top-down modulation facilitating reflex responses as discussed above. Increased excitability of the entire spinal system might explain that the distraction task facilitated the NWR with no distinction between stimulation site or between muscles.

RVM on/off cells might be playing a key role as the output of the PAG-RVM system, shifting the balance to pro-nociceptive spinal states, as previously seen in animal studies [189], [191]–[193].

The facilitation of the NWR might be further explained by a reduction of tonic descending control when the attention of the subject is drawn from the stimulus into another cognitive demanding task. The distraction condition of Study III might resemble that of a subject being unaware of an upcoming noxious stimulation. In this regard, Liebermann and Defrin showed that when subjects were engaged in a task and did not expect a stimulus, the latency of the NWR was significantly reduced [202]. In our baseline condition, subjects were aware that a series of potentially noxious stimuli were to be delivered. This might have kept or enhanced a tonic descending inhibition over the NWR, explaining the difference between distraction vs baseline and distraction vs attention of Study III.

Attention to the stimulation, on the other hand, did not effectively modulate the magnitude of the NWR. There was no significant difference in the magnitude of the NWR between the attention and the baseline conditions, regardless of the site stimulated and the recorded muscle (TA and BF). The lack of modulation during the attention task seems to be related to a limitation of the methodology. The task consisted in localizing the stimulated site. As only two stimulating electrodes were used in Study III, the task was relatively easy to complete (as seen in the low number of errors 7%). With a cognitive task too simple, the cognitive demand was likely not enough to induce an effective modulation of spinal nociception.

3.2. DIFFERENTIAL MODULATION OF SPINAL AND SUPRASPINAL OUTCOMES

It is important to note that in Study III and on those reported by Bjerre et al. [182] and Arguissain et al. [203], pain intensity ratings were not reported. In our study, this outcome was excluded due to methodological limitations. Including a rating task after each stimulation would jeopardize the condition of distraction, since rating the pain requires the subject to focus on the stimulus and the stimulated area. Albeit this limitation, the available literature seems to be consistent regarding the inhibitory effect of distraction on pain ratings [199], [200], [204], [205]. During Study III, participants were instructed to perform a Stroop test, while the NWR was elicited and recorded (see detailed methodology in the full article [3]). Other studies have previously used a modified Stroop test, similar to the test used in Study III, as a distracting methodology [199], [200], [206]. According to the literature, distracting participants from the stimulus seem to induce some form of endogenous analgesia, and therefore, significantly reduces pain perception. Based on that evidence, one might expect that the NWR being a proxy of spinal nociception, will be modulated in

the same direction. On the contrary, the results of Study III showed significant facilitation of the NWR when subjects performed the distracting task (compared to baseline and the attention task). In the light of the evidence just presented, it seems that spinal (NWR) and supraspinal (pain intensity) responses can be differentially modulated by cognitive tasks involving attentional shifts.

The mechanisms behind that differential modulation are still to be further clarified. As introduced in Chapter 1, nociceptive information is conveyed to the dorsal horn of the spinal cord through A and C nociceptive afferent fibers. These two classes of fibers have different conduction velocities and participate in the encoding of different dimensions of the pain experience. An illustrating example is the so-called first and second pain, each driven by activity in Aδ and C fibers, respectively. Evidence from animal studies has shown that the PAG-RVM system can differentially modulate Aδ vs C fiber mediated nociception [207], [208]. How this modulation is implemented in the SC is still being investigated [193], [209]–[211]. A simplified model that might explain how the PAG differentially modulates A and C fiber-driven nociception was proposed by Waters and Lumb [211], and further expanded by Heinricher and colleagues [193]. The latter authors proposed that the modulation from the PAG results in inhibition on a certain DDH neuron that depends on to what degree it receives C-fiber input (most of which relayed through the superficial layers) [193], [211]. According to that model, DDH neurons receiving multiple inputs from C-receptive superficial neurons (C (+)) will be strongly inhibited. On the other hand, DDH neurons that receive few(non) input from superficial C-receptive neurons (C (-)), are less(not) inhibited [193]. The NWR recorded in Study III was quantified in a reflex window that reflects Aδ driven responses. Therefore, it is possible to speculate that a differential modulation of Aδ and C driven nociceptive by the PAG-RVM system (see Figure 3), might play a role in the facilitation of the NWR observed in Study III, together with the inhibition of perceived pain intensities reported in the literature.

Finally, another contribution that might be behind a differential modulation during distraction is from an increased α-motoneuron excitability induced by activation of the PAG. Evidence of the latter has been recently reported showing an increased muscle tone driven by activation of ventrolateral PAG associated with survival behavior [212].

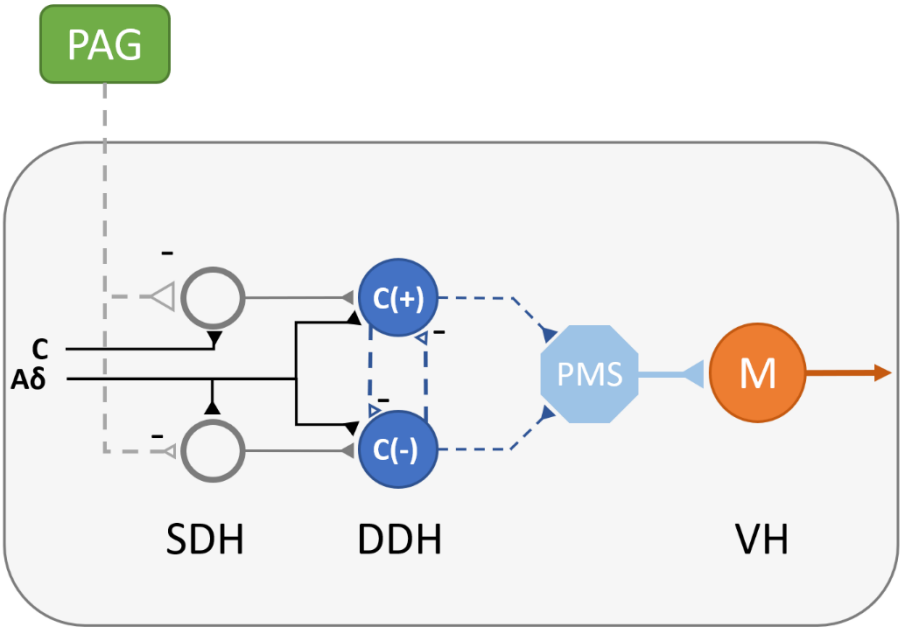

Figure 3: A schematic model of a spinal cord circuit that might explain the facilitated NWR observed during the distraction condition. Nociceptive primary afferents (C/Aδ) enter the DH of the SC and synapse with superficial dorsal horn (SDH) neurons (gray circles). The NWR in the present project was quantified in a time window congruent with Aδ conduction velocity. Blue circles represent DDH neurons that are primarily responsive to peripheral C and Aδ fiber activation, C(+) and C(-) respectively [193], [211]. *Descending modulation from the PAG induces its modulatory effect primarily on the SDH, although some direct projections to the DDH exist (not shown). According to the model proposed by Waters and Lumb* [211], *segmental inhibition between DDH neurons likely plays a role in the facilitation of Aδ driven activity. Through a circuit of interneurons that coordinate the reflex response (PMS: Premotor system), information can reach alpha-motoneurons (M) for the recruitment of specific muscles. Although DDH neurons in the reflex arch are not projecting to supraspinal centers* [97], *information that might lead to the perception of pain is transmitted through the DDH and ascend in the STT (not shown). See the text for discussion and references.*

CHAPTER 4. LIMITATIONS

The experimental studies behind the present project are not free from methodological limitations. First, findings from animal studies using direct recordings of central neurons are of importance in the understanding of mechanisms behind nociception. The translation of those relevant findings to humans is limited by the fact that only non-invasive techniques can be used, such as the quantification of the NWR. On the other hand, it is also worth mentioning that human studies have the advantage that self-reports can be obtained from the participants. Although subjective, perceptual reports complement the multidimensional assessment of the pain experience.

Second, the three studies behind this project used electrical stimulation to elicit the NWR. The electrical stimulus is an artificial stimulus that simultaneously depolarizes A and C fibers. All the studies behind this project quantified the NWR in a time window compatible with Aδ afferents mediating the observed responses. Due to the slow C fibers' conduction velocity, their contribution is likely not affecting the observed responses. On the other hand, conduction velocity of Aβ fibers is faster than Aδ, and therefore their contribution cannot be completely discarded. It is important to note, however, that according to a previous computational model [213] reducing the diameter of the stimulating electrode (as done in this project), favors the recruitment of Aδ fibers.

Third, as seen in the first chapter, the NWR involves more than two muscles. Although the inclusion of several lower limb muscles was regarded as unnecessary for assessing the hypothesis behind Study I, II, and III, the complete biomechanical assessment and specific contribution of different muscles might be of value for future research.

Last, including pain ratings on the third study, could have been useful to directly assess if the modulation induced in the NWR and in the perception of pain intensity are differentially modulated by the distracting task. As stated previously, it was not included to prevent confounding the interpretation of the data. A different design in which assessment of pain intensity is made by blocks of stimuli could have been an alternative approach.

CHAPTER 5. SYNTHESIS

The results of Study I [1] showed that the NWR spinal pathway integrates spatial information of the nociceptive stimulation. Spatial summation was observed on the NWR recorded in TA muscle and on perceived intensities. The summation was sub-additive suggesting that inhibitory mechanisms also play a role. By separating simultaneous stimuli, the NWR was inhibited while the perception of pain seemed to increase. In particular, when simultaneously stimulating the medial and lateral sides of the sole of the foot, a relative inhibition of the NWR was observed. The decrease in the magnitude of the NWR, as discussed in Chapter 2, is likely explained by the defensive role of the NWR implemented in its modular organization where individual RRF serve different purposes in the withdrawal reaction.

In Study II [2], evidence suggested that a temporal delay incorporated between simultaneous stimulation is modulating the NWR but not the perceived intensities. Proximal (TA) and distal (BF) muscles were differentially affected by the length of the delay, suggesting that a complex tempo-spatial integration, involving different segments of the spinal cord is found in the NWR pathway. Although the perceived intensities were not significantly affected by the temporal delay, other perceptual outcomes were. Specifically, the discrimination of the sequential stimuli was significantly facilitated when increasing the temporal delay and when stimuli were delivered in different sites (rather than in the same site). This likely suggests that spatial information is involved in the temporal discrimination of sequential stimuli. This study also confirmed SS on the NWR, similarly as in Study 1.

Finally, Study III [3] provided further evidence that a purely cognitive task can engage top-down modulation over spinal nociceptive processing. The PAG is most likely involved in the modulation of spinal nociception due to the distracting task used in Study III. A net facilitation of the NWR was observed during distraction that can be explained by the activity of a specific group of neurons in the RVM that enhance spinal nociception. Interestingly, the same distracting task used in this project was shown to induce analgesia in other human studies. The differential modulation of reflex and perceptual responses to noxious stimuli might be functional to the defensive role of the NWR to prevent tissue damage. The spatial summation due to simultaneous stimulation was not different between baseline and distraction, suggesting that the mechanisms behind spatial integration are not under cognitive descending control.

5.1. A DEFENSIVE PERSPECTIVE

Most of the results observed in the three studies included in this project can be explained from a reflex defensive perspective. Spatial summation (repeatedly observed in the three studies) has a clear defensive role since it facilitates withdrawal when the stimulated area (and thereby the magnitude of the threat) increases. The summated perceived intensities would also alert the subject that the noxious stimulus might have increased its potential to harm the body. Interestingly, Study I showed that the simultaneous stimulation of the medial and lateral sides of the sole of the foot reduced the magnitude of the NWR. Although a priori it seems like a paradoxical inhibition (due to the observed distance-based SSP), it is coherent with the optimal motor response that likely withdraws the exposed tissue (entire sole of the foot) from the stimulus. This is inhibition of fine-tuning distal muscles that serve inversion of the foot to potentially stabilize the talocalcaneal joint to withdraw the entire foot. Simultaneous stimulation and small temporal delays were generally perceived as a single stimulus (Study II), and in those cases, the BF-NWR was indeed facilitated. When the temporal delay was increased, the magnitude of the TA-NWR was facilitated, while the opposite pattern was observed in the BF-NWR.

Finally, regarding the effect of the distraction task on the NWR investigated in Study III, previous evidence has shown that the descending modulation by the PAG can differentially modulate afferent A and C fiber-driven nociception [210], [211]. Additionally, recent evidence has shown that the PAG can simultaneously and differentially affect sensory and motor systems [209], [214]. Reducing nociceptive inflow into supraspinal structures at the spinal cord level has been recognized as an efficient means for survival behavior [211], [215], [216]. Distraction induced by a highly demanding cognitive task might induce modulation from the PAG-RVM system compatible with the results obtained in Study III. During the distraction condition, subjects were instructed to prioritize the correct execution of the cognitive task. One might speculate that in those conditions that participants assign their cognitive resources to the distracting task, it is desirable to facilitate defensive motor responses to preserve homeostasis. As discussed above, the PAG-RVM system is potentially capable of facilitating fast defensive reflex responses (Aδ-fiber driven) while inhibiting input from C-fiber activation that might act as a survival-distracting factor [193], [209]–[211].

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