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BY
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Lea holds a B.Sc. and M.Sc. in Biomedical Engineering and Informatics from Aalborg University. After obtaining the M.Sc. she enrolled as a Ph.D. student under the supervision of Winnie Jensen in the Neural Engineering and Neurophysiology group, and as part of Center for Neuroplasticity and Pain (CNAP) which is supported by the Danish National Research Foundation (DNRF121). Co-supervision is by Ernest N. Kamavuako, King's College, London UK.

She has been involved in the teaching and supervision of several semesters at Biomedical Engineering, Clinical Science and Technology, and Medicine. Furthermore, the project and results of the PhD work have been disseminated through poster and oral presentation at the Scandinavian Association for the Study of Pain (SASP) annual conference, and at the annual seminar for the Center for Neuroplasticity and Pain at Aalborg University.

ENGLISH SUMMARY

Chronic pain is a global issue and subject to enormous research interest, but remains poorly understood due to its inherent complexity, the heterogeneity in symptom development among patients, and the lack of objective measures to assess pain. With animal models of chronic pain conditions, it is possible to study specific mechanisms in a highly controlled environment. Typically, pain is studied in the sub chronic or chronic phase of pain. However, several studies using these models have shown that functional cortical changes are already present days or weeks after an intervention, which suggests that a neurophysiological change must happen before this point in time.

The aim of the present PhD work was therefore to investigate functional cortical changes in the processing of cortical activity and interactions in the acute phase after a peripheral nerve injury in an animal model of neuropathic pain.

This thesis is based on three original scientific studies. In **Study I**, changes in the characteristics of electrically evoked cortical potentials following the spared nerve injury model was investigated. The results showed changes in both amplitude and latency of the accumulated spiking activity in the primary sensory cortex and anterior cingulate cortex. **Study II** analyzed the functional connectivity between primary sensory cortex and anterior cingulate cortex during electrical stimulation, and how these interactions changed following the same animal model of pain. This study also demonstrated early cortical changes such as a stronger interaction between the primary sensory cortex and the anterior cingulate cortex in the hours following injury. In **Study III**, spontaneous cortical activity was analyzed before and after intervention with the pain model. The results showed that the model of pain led to a decreased spontaneous information flow between the anterior cingulate cortex and the primary sensory cortex.

The overall conclusion of this thesis is that cortical functionality is affected as early as a few hours after a peripheral nerve injury. The evoked activity seems to change in a way similar to hyperalgesia and allodynia mechanisms–such as seen in human neuropathic patients–with an increased response to both noxious and non-noxious stimuli. Contrarily, the changes in spontaneous cortical functionality are in the opposite direction, indicating that other mechanisms or cortical areas take over after injury. This work contributes to current knowledge by showing cortical alterations resulting from a peripheral nerve injury in a time frame shorter than previously investigated.

DANSK RESUME

Kronisk smerte er en global udfordring og grundlag for meget forskning. Alligevel er det stadig i dag ikke fuldt forstået grundet dets komplekse natur, forskelligheden i symptomudvikling hos patienter og manglen på objektive målemetoder til at kvantificere smerte. Med dyremodeller af kroniske smertetilstande er det muligt, at studere specifikke mekanismer i et kontrolleret miljø. Typisk undersøges smerte i en sub-kronisk eller kronisk fase. Der er imidlertid flere studier der har vist, at funktionelle kortikale forandringer er tilstede allerede dage eller uger efter en intervention, hvilket indikerer, at neurofysiologiske ændringer sker inden dette tidspunkt for målingen.

Formålet med denne PhD var derfor at undersøge funktionelle kortikale ændringer i hjerneaktivitet og interaktioner mellem hjerneområder i den akutte fase efter en perifer nerveskade i en dyremodel af neuropatisk smerte.

Denne afhandling er baseret på tre originale videnskabelige studier. I **Studie I** blev karakteristika af elektrisk-evokerede kortikale potentialer efter ”spared nerve injury” undersøgt. Resultaterne viste, at der er ændringer i både amplitude og latenstid af det akkumulerede højfrekvente aktivitet i primær sensorisk kortex og anterior cingulate kortex. **Studie II** analyserede den funktionelle forbindelse mellem primær sensorisk kortex og anterior cingulate kortex under elektrisk stimulation, og hvordan disse interaktioner ændres efter intervention med samme dyremodel af smerte. Dette studie demonstrerede også tidlige kortikale forandringer i timerne efter nerveskade. Denne interaktion fra det primære sensoriske kortex til det anteriore cingulate kortex var øget, hvilket også er vist i tidligere studier. Dog er det aldrig tidligere vist, eller undersøgt, i den akutte tidsramme som gjort her. I **Studie III** blev spontan kortikal aktivitet analyseret før og efter intervention med smertemodellen. Resultaterne viste at smertemodellen førte til et mindsket spontant informationsflow mellem anterior cingulate kortex and primær sensorisk kortex.

Den overordnede konklusion på denne afhandling er, at den kortikale funktionalitet er påvirket så tidligt som få timer efter perifer nerveskade. Den evokerede aktivitet forekommer at blive ændret på en måde der ligner hyperalgesi og allodyni –som set ved mennesker som lider af neuropatisk smerte- med en øget respons på både høj- og lavintensitets stimuli. Den spontane kortikale funktionalitet ændres i en komplet modsat retning (mindskes), hvilket indikerer, at andre mekanismer eller kortikale områder overtager efter nerveskade. Dette arbejde bidrager til den nuværende viden ved, at vise hvordan kortikale ændringer, resulterende fra perifær nerveskade, sker i en langt kortere tidsramme en hidtil vist (og undersøgt).

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CHAPTER 1. INTRODUCTION

Pain is a complex subjective experience and it is influenced by many genetic, psychosocial, and other factors (Ploner, Sorg and Gross, 2017). Previous experiences, expectations (Wiech, 2016), and day-to-day variations such as sleep and temperature may influence the experience of pain. Notably, acute pain is in itself not dangerous– it is a defense mechanism to protect the body from getting injured or to ensure rest in case of injury or disease. In some cases, however, the acute pain persists longer than necessary to protect the body. When studying pain, chronic pain is often implied although an understanding of the acute phase is not achieved in many cases. This is also the case in neuropathic pain, where the acute phase has recently been receiving increasing attention due to this issue (Hansson, Baron and Stubhaug, 2019).

Neuropathic pain is a condition with large economical, societal, and individual consequences. It is estimated that 7-10 % of the population suffer from this type of chronic pain (Bouhassira *et al.*, 2008; Dworkin *et al.*, 2013; Scholz *et al.*, 2019). These patients have lower quality of life than the general population (Schmader, 2002); some are not capable of having a normal job (Scholz *et al.*, 2019) and treatment and medication are often insufficient (Scholz *et al.*, 2019). Furthermore, there is a high prevalence of depression among chronic neuropathic pain patients (Schmader, 2002; Toth, Lander and Wiebe, 2009). One of the issues is that there is currently no way of predicting who will develop chronic neuropathic pain. Thus, it is not possible to establish a baseline before development of neuropathic pain in human subjects. Without a baseline it is difficult to study underlying pain mechanisms as there is often large variation between subjects. Alternatively, animal models of pain enable the possibility to record a baseline before the animals are subjected to a model of neuropathic pain. Furthermore, when using animal subjects, it is possible to record directly from the cortical units thereby providing unique insights into the cortical mechanisms behind pain processing.

Several studies have been conducted using animal models of neuropathic pain investigating functional cortical changes showing that changes do occur days or weeks after injury (LeBlanc *et al.*, 2014, 2016; Chao, Chen and Yen, 2018; Chen *et al.*, 2018; Singh *et al.*, 2020). Changes occurring within the first day following an injury are however a black box and thus the development in the early acute phase is unknown.

The focus of this thesis was to study the early development of functional cortical changes in an animal model of neuropathic pain.

CHAPTER 2. STATE-OF-THE-ART

2.1. NEUROPATHIC PAIN

Neuropathic pain was included in the ICD-11 in 2019 (Scholz *et al.*, 2019) and is thereby now perceived as an independent diagnosis and not only as a symptom in other diseases. The International Association for the Study of Pain (IASP) has defined it as;

> *"Pain caused by a lesion or disease of the somatosensory nervous system."*
> (International Association for the Study of Pain, 2017)

It is estimated that up to 10 % of the general population experience neuropathic pain (Van Hecke *et al.*, 2014; Scholz *et al.*, 2019). Like many other painful conditions, neuropathic pain results in a decreased quality of life (Beniczky *et al.*, 2005; Mcnicol, Midbari and Eisenberg, 2013). The condition remains difficult to manage due to lack of effect or severe side effects of treatment (Beniczky *et al.*, 2005; Mcnicol, Midbari and Eisenberg, 2013; Scholz *et al.*, 2019).

2.2. NEUROPHYSIOLOGY OF NEUROPATHIC PAIN

Pain is regulated both in a bottom-up and top-down fashion (Ploner, Sorg and Gross, 2017); bottom-up by peripheral nerves or neuromas spontaneously firing or being more sensitive to stimuli (Seifert and Maihöfner, 2011), and top-down by cognitive factors and descending pain control systems (Heinricher *et al.*, 2009). Cortical areas use neurotransmitters to excite or inhibit different neurons as a descending control (López-Álverez, Redondo-Castro and Navarro, 2019). In a condition of chronic neuropathic pain, both top-down and bottom-up mechanisms are affected. Molecular and cellular changes increase the excitability in both injured and uninjured peripheral neurons while top-down inhibitory mechanisms are decreased (López-Álverez, Redondo-Castro and Navarro, 2019).

In chronic pain conditions, central sensitization may sustain the perceived pain after the initial injury or disease has disappeared causing the acute pain to continue into chronic pain (Seifert and Maihöfner, 2011). Central sensitization is a change in sensory response so that the neural system becomes more sensitive to pain (Woolf, 1991; Latremoliere and Woolf, 2009). In addition to peripheral and spinal consequences of pain, it is evident that cortical changes appear. These changes can be within one specific area or in the interactions between areas. A combination of cortical areas has for many years been referred to the neuromatrix (Melzack, 1999). However, it has been shown that the activation of these areas is not pain-specific (Iannetti and Mouraux, 2010). Among the areas found to be activated in acute pain in healthy

patients (painful stimuli) and chronic neuropathic pain patients (pain >3 months) are primary and secondary somatosensory cortex (SI and SII), anterior cingulate cortex (ACC), insular cortex, pre-frontal cortex (PFC) and thalamus (Apkarian *et al.*, 2005; Geha and Apkarian, 2005). These areas are also found to be activated in studies using animal models of pain (Thompson and Bushnell, 2012). While nerve injury causes immediate reorganization in brainstem, changes in the cortex are believed to take weeks to months (Navarro, Vivó and Valero-Cabré, 2007). Even though reorganization in the cortex may not happen immediately, the cortical activation may be altered by activation of silent synapses (López-Álverez, Redondo-Castro and Navarro, 2019). Fast changes could also include decreased inhibition and changes in conductance and receptors both in the cortex and sub-cortex (López-Álverez, Redondo-Castro and Navarro, 2019).

In neuropathic pain, there is often not a clear relationship between the intensity of a stimulus and the resulting pain sensation and there is often spontaneous pain sensation without prior stimulation after nerve injury (López-Álverez, Redondo-Castro and Navarro, 2019). Many neuropathic pain patients experience either hyperalgesia, which is an elevated response to noxious stimuli, or allodynia, which is when a non-noxious stimulus is perceived as noxious (IASP, 1994; Scholz *et al.*, 2019).

Cortical neurons are constantly inhibiting or exciting other neurons as a way of processing incoming information (Ploner, Sorg and Gross, 2017). When these processes become synchronized in groups of neurons, it can be measured as neural oscillations. In regards to pain, altered cortical oscillations have been related to pain processing although similar to the neuromatrix, no one oscillatory frequency is pain-specific (Ploner, Sorg and Gross, 2017). Of special interest are the gamma oscillations, as they have been found to correlate with pain in healthy subjects during noxious stimulation (Gross *et al.*, 2007; Schulz *et al.*, 2015). Similar results have been found in rats (Wang *et al.*, 2016; Peng *et al.*, 2018). In chronic pain patients, increased theta and beta oscillations have been linked to pain (Sarnthein *et al.*, 2006; Stern, Jeanmonod and Sarnthein, 2006), which are also supported by rat studies (Cao *et al.*, 2016; Chen *et al.*, 2018; Song *et al.*, 2019).

2.3. ANIMAL MODELS OF NEUROPATHIC PAIN

Human studies of pain have two major limitations: they have to be conducted either on patients with pain, eliminating the possibility of having a pain-free baseline, or they use a short-term (surrogate) model of pain.

When using animal models of pain, chronic, irreversible injuries can be used as interventions while recording symptoms before and after. Although this enables studying the time-course of pain-development, these models are only mimicking some of the symptoms of the chronic disease they are supposed to model (Gregory *et al.*, 2013).

Several animal models of neuropathic pain have been developed and used. They all have in common that they do not completely reflect the condition but merely mimics some of the symptoms (Berge, 2011; Gregory *et al.*, 2013) such as mechanical allodynia and thermal hyperalgesia for days (Xie *et al.*, 2005; Djouhri *et al.*, 2012; LeBlanc *et al.*, 2014; Gerard *et al.*, 2015; Chang *et al.*, 2017), weeks (King *et al.*, 2011; LeBlanc *et al.*, 2016; Chen *et al.*, 2018; Singh *et al.*, 2020), or even months (Decosterd and Woolf, 2000) after injury. These models are the chronic constriction injury (CCI), crush injury, spared nerve injury (SNI), denervation, and spinal ligation (Figure 1). Most of the models are irreversible. Additionally, many of these studies have shown cortical changes following injury.

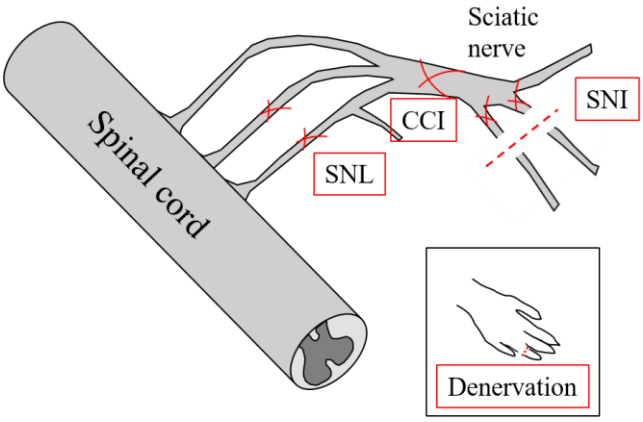

Figure 1: Illustration of the different animal models of neuropathic pain: the spinal nerve ligation (SNL) model, chronic constriction injury (CCI) model, the spared nerve injury (SNI) model, and forepaw denervation.

2.4. **ASSESSMENT OF PAIN IN ANIMALS**

Pain is a subjective feeling and one major drawback of animal studies is of course that the animals are not capable of describing their subjective perceptions. Thus, the assessment of pain in animal studies are indirect assessments of mechanisms related to pain perception. The assessments are often assuming either increased sensitivity as a result of the pain model or anatomical or functional changes e.g. in the brain. All measures are indirect and based on assumptions about changes following injury or disease but can be used to confirm the presence or absence of specific mechanisms. The two groups of assessment of pain in animal studies include behavioral assessments and objective measures. In this work, the focus has been on cortical changes, which can be assessed using imaging or electrophysiology.

Behavioral assessments include withdrawal reflexes, paw licking and the Grimace scale. These assessments provide information about sensitivity to external or internal

stimuli. Using this type of assessment, thermal hyperalgesia and mechanical allodynia have been shown after CCI (Xie *et al.*, 2005; Gerard *et al.*, 2015) and SNI (Decosterd and Woolf, 2000; Xie *et al.*, 2005).

In both subjective and objective measures, presumed noxious and non-noxious stimuli are used. An objective measure of whether a stimulus is noxious or non-noxious is conduction velocity which can be used to investigate if nociceptive fibers are activated. A previous study has used conduction velocity to estimate which types of fibers and how many are recruited at specific stimulation intensities of electrical stimulation. At two-times motor threshold, Aβ fibers are recruited and around 50 % are activated (Chang and Shyu, 2001). When increasing the stimulation intensity from two- to ten-times motor threshold an increasing amount of Aβ fibers are recruited. Aδ fibers are recruited at intensities above five-times motor threshold. At ten times motor threshold, around 70 % of Aδ and possibly few C-fibers are activated in addition to most Aβ fibers (Chang and Shyu, 2001).

In the imaging studies, the most commonly used method is functional Magnetic Resonance Imaging (fMRI). In these studies, activation of cortical areas following pain models or nociceptive stimuli can be shown. Several studies have shown changes in cortical areas such as SI, ACC, insula, and amygdala after peripheral nerve injury (Han *et al.*, 2013; Chao, Chen and Yen, 2018; Onishi *et al.*, 2018). Additionally, nucleus accumbens and its interaction with other areas have been shown to change following SNI (Chang *et al.*, 2014, 2017). The advantage of using fMRI in animal models of pain is that it is non-invasive and it is therefore also used in human pain studies, increasing the translation. The disadvantage is the low temporal resolution compared to electrophysiological measures.

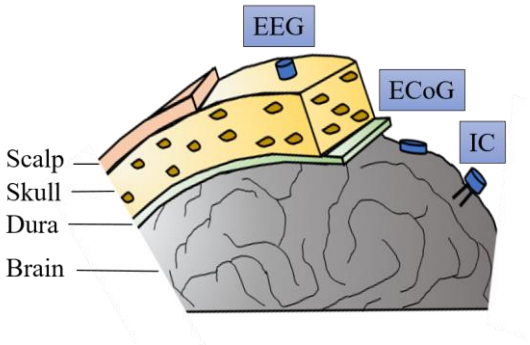

Figure 2: Illustration of the placement of different electrodes for electrophysiology: Electroencephalography (EEG), electrocorticography (ECoG), and intracortical multi-electrode array (IC MEA)

Electrophysiological measures can be recorded at different levels of the cortex from electroencephalography (EEG) outside the cortex, electrocorticography (ECoG) on the surface of the cortex, and single- or multiunit recordings recorded intracortically (IC, Figure 2) to spinal cord recordings and peripheral nerve recordings using e.g. a cuff electrode. Using patch-clamps, the purpose is similar to recording intracortical signals. These types of studies, i.e. electrophysiological, are used to show changes in cortical activity as an objective parameter of pain assessment. Both EEG and ECoG studies have shown increased power (amplitude of oscillatory activity) in SI and PFC (only EEG) following CCI (LeBlanc *et al.*, 2014, 2016). IC studies are typically used to show changes in spatial specific areas and in some cases on specific levels in cortical areas. As the IC studies are spatially limited, the results are also limited to a defined area. Mixed results in studies using animal models of neuropathic pain show no change in thalamus activation (LeBlanc *et al.*, 2014), increased insula and SI activation (Chao, Chen and Yen, 2018), specifically in layer 5 of SI (Han *et al.*, 2013), and that SI activation increases the activation of ACC following injury (Singh *et al.*, 2020). Finally, using patch-clamp whole-cell recordings from ACC, it was shown that activity increased after CCI (Chen *et al.*, 2018). Using peripheral nerve recording or recording from dorsal root ganglion (DRG) neurons, it was shown that C and Aδ nociceptive fibers had increased spontaneous (ectopic) firing after peripheral or spinal nerve injury (Xie *et al.*, 2005; Djouhri *et al.*, 2012).

2.5. ANALYSIS OF CORTICAL ACTIVITY

When using electrophysiological recordings, two types of analysis may be used providing two different types of information. With IC single- or multiunit activity, the spikes, either spontaneous or event-related, are analyzed. The other option is oscillatory activity from local field potentials (LFP) from IC recordings, EEG, or ECoG (Figure 3).

2.5.1. DATA PROCESSING APPROACHES

Several approaches can be taken when analyzing electrophysiological data. Overall, three methods are traditionally used either alone, or in combination. In the analysis of spikes from either IC or ECoG, analyses in the time domain contribute with information about activity in certain time intervals, such as the latency of peak activity. With both EEG and LFP's from IC or ECoG, the frequency domain is often explored. In this type of analysis, the signals are filtered into specific frequency bands, often in the 1-200 Hz range (Song *et al.*, 2019; Tan *et al.*, 2019; Guo *et al.*, 2020). The frequency-domain analysis can be used to explore changes in oscillatory frequency, shown as in- or decreases in activity in different frequency bands. The frequency bands traditionally used are δ (1-4 Hz), θ (4-8 Hz), α (8-14 Hz), β (14-40 Hz), and γ (40-100 Hz), sometimes divided into γ (40-49 Hz), and high-γ (50-100 Hz, Figure 3) (Noachtar *et al.*, 1999). In combination with one of the other types of analyses, changes in the spatial domain can be investigated when recording from more than one

electrode. In this type of analysis, spatial changes in peak activity and changes in communication between cortical areas can be explored.

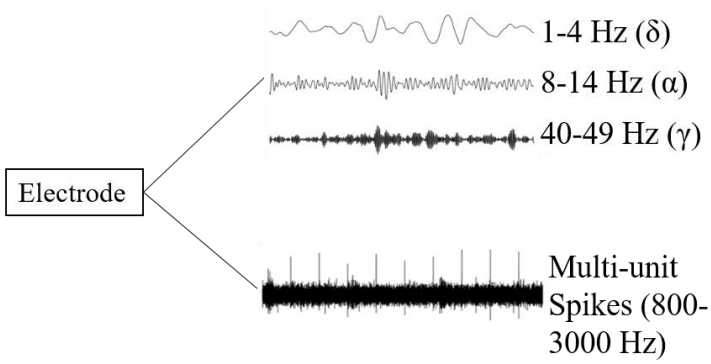

Figure 3: Illustration of local-field potentials filtered into the δ, α, and γ frequency bands, and multi-unit spiking activity from the same signal.

2.5.2. SPIKING ACTIVITY

Spiking activity can be recorded as either single- or multi-unit activity. The spiking activity is often processed in a frequency range up to 3000 Hz (Wang *et al.*, 2003, 2011; Yang, Shih and Shyu, 2006). When recording multi-unit activity, spike sorting can be done, thus analyzing single-unit activity. Alternatively, the multi-unit activity can be analyzed as a cluster of activities. When analyzing single- or multi-unit activity, the spikes or action potentials from the neurons are measured. The spiking activity changes when inhibiting or exciting mechanisms are exerted on the neurons making them fire less or more.

Results from previous studies in animals models of pain

Extensive research has shown that spiking activity in SI and ACC increases as a result of stimulation with laser (Kuo and Yen, 2005; Xiao *et al.*, 2019), electrical (Yang, Shih and Shyu, 2006; Shyu, Chen and Shih, 2008; Wang, Zhang, *et al.*, 2008; Ma *et al.*, 2016), or mechanical (Wu *et al.*, 2012; Singh *et al.*, 2020) noxious stimuli. Additionally, some have found that ACC in some cases is only activated with noxious stimuli (Yang, Shih and Shyu, 2006; Wu *et al.*, 2012) and the latency of activation is longer compared to SI (Kuo and Yen, 2005; Wang, Chang, *et al.*, 2008). Finally, similar results have been found when using the CFA model of inflammatory pain (Singh *et al.*, 2020) or forepaw denervation (Han *et al.*, 2013) instead of only noxious stimulation.

2.5.3. LOCAL FIELD POTENTIALS

The analysis of LFP's is a measure of the frequencies at which neurons are firing, which is similar to the analysis of EEG as it is based on oscillatory activity in the low frequency range. In other words, it is a measure of pathways opening or closing. There are overall three types of studies in this field: recordings of cortical activation during noxious stimuli, recordings of spontaneous cortical activation in resting-state after intervention by a model of pain, and recordings of cortical activation during noxious stimuli following a model of pain.

Results from previous studies in animal models of pain

During noxious mechanical, laser, or electrical stimulation, previous studies have found, among other areas, increased delta and theta oscillations in ACC (Li *et al.*, 2017; Shen *et al.*, 2017; Zhang *et al.*, 2018), decreased alpha and beta, and increased gamma oscillations in ACC and SI (Li *et al.*, 2017). This research shows a trend towards an increase in both very low-frequency and high-frequency oscillatory activity within SI and ACC. Resting-state activity is usually investigated days or weeks after an injury. Only few studies have investigated the resting-state oscillatory activity. LeBlanc et al. (2014) showed increased theta oscillatory activation of SI and thalamus following the SNI model both a few days and two weeks after SNI. Chen et al. (2018) recorded from ACC and showed increased delta, theta and gamma oscillatory activation weeks after CCI. In continuation of the resting-state studies, evoked activation can be used to investigate the whole system from the peripheral stimulation site to the cortical area being recorded. In a model of inflammatory pain (CFA), the gamma oscillations in SI were increased after intervention and the cortical activity correlated with hyperalgesia (withdrawal from laser stimuli) (Wang *et al.*, 2016). In the same model of inflammatory pain, the gamma and theta oscillatory activation was also found to increase in ACC during laser stimuli (Zhang *et al.*, 2018). Han et al. (2013) recorded an increased activation of SI during electrical stimulation 1 hour after forepaw denervation.

2.5.4. FUNCTIONAL CONNECTIVITY

In addition to investigating the oscillatory activation of one or several cortical areas, the interaction of relevant areas has been investigated. Similar to the studies mentioned above, two different types of studies have been conducted: resting-state connectivity studies and evoked connectivity studies. The time frame in these studies differ from minutes after an injury to several weeks. Connectivity analysis is a family of signal processing methods aiming to investigate the relationship between two groups of neurons. This connectivity can be anatomical through the neurons connecting cortical areas or functional through a relationship in activation where the latter was used in this work. According to the gating theory, neurons open and close the pathway of communication with different frequencies in the sending and receiving end (Salinas and Sejnowski, 2001; Fries, 2005). The temporal coordination is

important for the efficiency with which this exchange of information is done (Salinas and Sejnowski, 2001). Correlations between neurons may be affected internally from neuronal populations (Lampl and Yarom, 1993) or driven by stimulation (Engel, Fries and Singer, 2001).

Traditionally, connectivity analysis has been utilized with two distinct approaches. When analyzing the temporal correlation or synchrony between groups of neurons, undirected connectivity (sometimes mentioned as functional connectivity) measures are used, and when analyzing how one group of neurons influences another, directed connectivity (sometimes mentioned as effective connectivity) measures are used (Friston, 1994, 2011).

Undirected connectivity

The undirected connectivity is a measure of time-locked amplitude trends of similarity of phase from two or more groups of neurons (Friston, 1994). Temporal correlations do not inform about direction of activity but may still unravel underlying plastic mechanisms (Singer, 1993). The most classic measure of connectivity based on the amplitude and time lag of signals is correlation (Nunez *et al.*, 1997). Correlation is the normalized covariance between two groups of neurons (Friston, 1994; Nunez *et al.*, 1997). The idea behind phase-based connectivity measures is that neural populations that are connected somehow will synchronize in their firing (Cohen, 2014). The phase-based measures use the phase angle differences, which are found by projecting the signals to the polar plane and finding the angle between the x-axis (real axis) and the point coordinates. When using magnitude squared coherence, the power of the signal is also taken into account (Cohen, 2014). Phase lag index (PLI) is less sensitive to outliers but does not consider large variation; that is if the values are very spread on the polar plot but still on the same side of the polar imaginary axis, the PLI value will be high (Cohen, 2014). If the PLI is close to 0 or pi on the polar imaginary axis, it can be suspected that it may be a result of volume conduction (Stam, Nolte and Daffertshofer, 2007).

Directed connectivity

Measuring directed connectivity implies causality although it cannot guarantee this because it only reveals a statistical relationship (Seth, 2010). Being based on regression models (Granger, 1969), directed connectivity measures can be used to investigate the direction of information (Friston, 1994). When using electrophysiology, directed connectivity is closely related to synaptic efficacy (Friston, 1994). The basis of Granger prediction or Granger causality is that if the prediction error of one time series decreases when including past measurement from another time series in addition to its own, the other time series can be said to predict that time series (Granger, 1969; Kamiński *et al.*, 2001; Seth, 2010).

Results from previous studies in animal models of pain

Short-term studies using noxious stimulation have shown correlated activation of SI and ACC in response to laser stimuli (Li *et al.*, 2017; Song *et al.*, 2019; Xiao *et al.*, 2019). The CFA model is used for both short term (hours) studies of the cortical response to pain and long term (days/weeks), whereas chronic models such as models of neuropathic pain are used long term (days/weeks). However, it is not investigated if the traditionally long-term models result in short term responses similar to those of short-term models. In the CFA studies, increased connectivity between SI and ACC during mechanical and laser stimuli has been found (Tan *et al.*, 2019; Guo *et al.*, 2020) similar to the results of the noxious stimuli studies.

Studies using chronic pain models are often hard to compare as they investigate different interactions and use models of different types of pain. However, one study found that the resting-state connectivity between thalamus and SI was decreased in several different models of neuropathic pain weeks after intervention (Zippo *et al.*, 2016). Days after a model of IBS, the resting-state connectivity was decreased between ACC and amygdala (Cao *et al.*, 2016). This was also found between SI and thalamus days and weeks after SNI (LeBlanc *et al.*, 2014; Zippo *et al.*, 2015, 2016) (see findings from previous studies using neuropathic pain models in Figure 4).

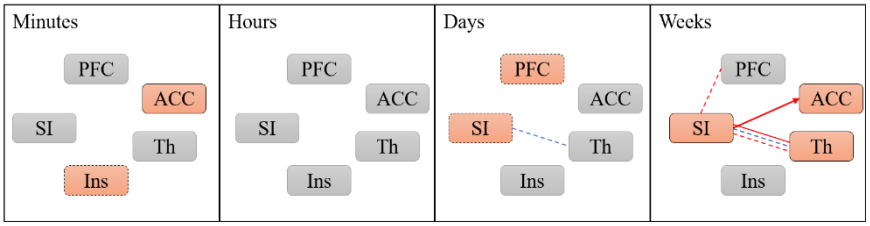

Figure 4: Findings from previous studies of animal models of neuropathic pain using electrophysiological measures in the minutes (Chao et al., 2018; Han et al., 2013), hours, days (Leblanc et al., 2014; LeBlanc et al., 2016), and weeks (LeBlanc et al., 2014, 2016; Singh et al., 2020; Zippo et al., 2015, 2016) after injury. Orange boxes/lines indicate increases and blue lines indicate decreases. Grey boxes indicates unreported or uninvestigated areas and connections. Dotted lines indicate resting-state studies and full lines, evoked potentials. Ins: Insular cortex, Th: Thalamus.

The relation between high-frequency spikes and low-frequency oscillation in animal models of pain have been explored. Spike-field coherence (SFC) is a measure of how accurate spikes follow LFPs with a specific frequency and might be related to cognition. By calculating the SFC, Cao et al. (2016) found that a model of IBS (inflammatory, not neuropathic pain model) disrupted SFC between ACC and amygdala.

CHAPTER 3. OUTLINE OF PH.D. WORK

Even though many questions regarding cortical processing of neuropathic pain remains to be answered, several things are clear. Many areas including SI, SII, ACC, insula, and PFC are somehow involved in the processing of pain and nociception. One of the reasons why the involvement of these areas are unclear is that there is not one area that is specifically activated by noxious stimuli. Cortical activation in the form of oscillatory frequency has been of increasing interest as several frequency bands have been found to be related to either self-perceived pain or stimulation intensity of a noxious stimulus.

In animals, pain and nociception are studied using high intensity, presumed noxious, stimuli or models of pain. The common feature of the models, regardless of which condition they model, is that they mimic some of the mechanisms that characterize the pain condition. Both noxious stimuli and pain models are evaluated using either observations of behavior, objective measures, or both. Behavioral measures have been used to show that models of neuropathic pain results in thermal hyperalgesia and mechanical allodynia.

These objective measures probe the nervous system peripherally, spinally, or cortically. The effect of neuropathic pain or neural injury may be in the form of changes in the number of neurons firing, firing rate or frequency, anatomical changes, or changed interactions between neurons or groups of neurons. Several of these changes can be quantified using electrophysiology. When using animal models of pain, it is possible to record directly from the cortex and thereby obtain fast changes of multi-unit (or single-unit) activity with a high temporal resolution. The traditional analysis of multi-unit activity is through high-frequency spiking activity or low-frequency LFPs.

As there is not one area or frequency band specifically activated by pain or by all types of pain and nociception, the interaction between cortical areas has gained research interest. Using different connectivity measures, both the undirected and directed functional connection can be studied.

Besides showing that the SNI model results in behavioral and cortical changes several days or even weeks after injury, not much is currently known about the neural changes after SNI, and especially in the acute phase. Days and weeks after injury there are increased activation of and interaction between the cortical areas involved in pain processing. It is not known how fast these changes appear and whether there is different activation within the first days compared to later changes. Before understanding the processing of neuropathic pain, the temporal gap that exists in the first hours after injury needs to be closed.

3.1. THESIS AIM

This thesis aimed to investigate the cortical response in an animal model of neuropathic pain in the first hours following injury.

3.2. SPECIFIC RESEARCH QUESTIONS

To address the thesis aim, the following specific research questions were formulated:

Q1. To what extent does the SNI model result in a short-term (hours) increased response to non-noxious (allodynia-like) and noxious (hyperalgesia-like) stimuli?

Q2. How does functional changes occur in the first hours after intervention by the SNI model?

Q3. How does the evoked interaction and the resting-state interaction between SI and ACC differ after SNI?

3.3. SOLUTION STRATEGY AND METHODOLOGICAL CHOICES OF THE THESIS

To evoke cortical changes, neuropathic pain must occur. This can be achieved using an animal model of neuropathic pain, mimicking the symptoms of neuropathic pain patients. In the studies conducted in relation to this thesis, the SNI model was used. The SNI model results in hypersensitivity to cold and mechanical stimuli (Baliki *et al.*, 2005; Chang *et al.*, 2014). By using the SNI model for several studies over many years it has been concluded that the model is reliable and robust because almost all rats develop the same symptoms (Pertin, Gosselin and Decosterd, 2012). In addition to behavioral studies, this model has been used in several studies investigating the cortical response to peripheral injury (Chang *et al.*, 2014, 2017; M. N. Baliki *et al.*, 2014; Chao, Chen and Yen, 2018).

Intracortical signals from SI and ACC were recorded before and after subjecting rats to the SNI model. With intracortical signals, a very high temporal resolution and no volume conduction (as seen with EEG) can be achieved. Furthermore, it enables both an analysis of spiking activity and analysis of LFPs, which are similar to EEG signals. The LFPs were used to perform connectivity analysis to explore the SI-ACC interaction, which combined with the IC recordings is a unique approach.

To avoid using two different modalities to induce non-noxious and noxious stimuli (some previous studies use e.g. brush and laser), electrical stimuli were used to evoke cortical potentials. With electrical stimuli, the intensity determinates whether it is noxious or non-noxious.

3.4. RESEARCH STUDIES

To address the research questions, three experimental studies were designed and conducted. **Study I** and **II** were designed to answer the first two research questions. **Study II** and **III** were designed to answer the second research question while a comparison of the results from **Study II** and **III** was assumed to answer the third research question. In all three studies, the SNI model was used as a model of neuropathic pain while recording intracortical signal from SI and ACC. The data were collected in one extensive experiment.

Study I: ***Tøttrup, L., Diaz Valencia, G.A., Kamavuako, E.N., Jensen, W., Modulation of SI and ACC response to noxious and non-noxious electrical stimuli after the spared nerve injury model of neuropathic pain.*** **Published in European Journal of Pain, 09 November 2020. doi.org/10.1002/ejp.1697**

In **Study I**, we investigated the amplitude and latency of the accumulated spiking response to noxious and non-noxious response. In addition, we subjected rats to the SNI model of neuropathic pain to investigate how the response would be altered. We hypothesized that the response in both areas would increase after injury and that the response in ACC would be slower than that in SI.

Study II: Tøttrup, L, Atashzar, S.F., Farina, D., Kamavuako, E.N., Jensen, W., ***Altered evoked low-frequency connectivity from SI to ACC following nerve injury in rats,*** **In preparation**

In **Study II**, we used the LFPs to investigate how the interaction between SI and ACC is altered by the SNI model. We used evoked LFPs to both noxious and non-noxious electrical stimulation as in study I. We hypothesized that the directed interaction from SI to ACC would be stronger than that from ACC to SI and that this interaction would be stronger following injury.

Study III: Tøttrup, L, Atashzar, S.F., Farina, D., Kamavuako, E.N., Jensen, W., ***Nerve injury decreases hyperacute resting-state connectivity between the anterior cingulate and primary somatosensory cortex in anesthetized rats,*** **Published in IEEE Transaction on Neural Systems and Rehabilitation, 25 November 2020. doi.org/10.1109/TNSRE.2020.3039854**

In **Study III**, we used only resting-state LFPs and their interactions between SI and ACC. We hypothesized that resting-state interactions were decreased immediately after SNI. The investigation of the interactions the following hours was more exploratory.

CHAPTER 4. METHODOLOGICAL APPROACHES

4.1. EXPERIMENTAL SETUP

All procedures conducted under this thesis was approved by the Danish Veterinary and Food Administration (J. no.: 2016-15-0201-00884). Nineteen rats were used in the studies, all delivered from Taconic, Denmark. At arrival at the facility, the rats were given two weeks to acclimatize to the environment. The rats were kept in cages with 2-3 animals in each and at a controlled temperature and humidity with a half-light/half dark cycle. Food and water were supplied ad libitum. Before any procedure, the rats were, on at least five different days, brought to the laboratory to get accustomed to the investigator and the anesthesia induction chamber. This was done to minimize the stress level at the day of the experiment as it may affect the cortical recordings and especially the stability of the anesthesia.

On the day of the experiment, the rats were anesthetized in an induction chamber with 4 % isoflurane vaporized in medical grade oxygen (99 %) at 2 L/min. After the initial anesthesia, the rats were placed in a mask in a stereotaxic frame (KOPF®) where the isoflurane was turned down to 2.5 % and supplied continuously at 0.5 L/min. The isoflurane was regulated between 1 and 2.5 % throughout the experiment based on the experimenter's assessment of heart rate, breath rate, and reflexes to paw and tail pinching. Several injections of saline were made to avoid dehydration. To ensure a stable temperature and avoid hypothermia, the rat was placed on a heating pad (ATC-2000, World precision instruments) controlled in a closed-loop system.

After the last recording, the rats were euthanized, by an intracardiac injection of pentobarbital which caused the heart to stop immediately.

4.2. SPARED NERVE INJURY MODEL OF NEUROPATHIC PAIN

An intervention with the SNI model was performed by ligating and transecting the tibial and common peroneal branch of the sciatic nerve while leaving the sural branch intact (Decosterd and Woolf, 2000). The purpose of leaving one branch intact is to avoid self-mutilation in recovery/survival studies (Devor and Raber, 1983). This procedure was therefore used to enable comparison with such studies.

4.3. INTRACORTICAL RECORDINGS

The cortical recordings were conducted using a multi-electrode array (MEA). This type of electrode records multi-unit activity from inside the cortex. The recordings were made using a TDT PZ2 preamplifier and a PZ5 NeuroDigitizer amplifier (TDT, Tucker-Davis Technology) and OpenX software (TDT) with a sampling frequency of 24.414 Hz.

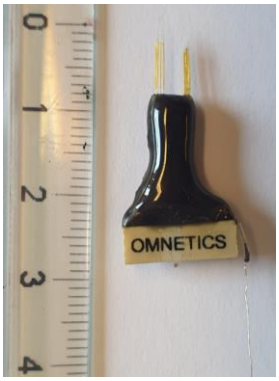

Figure 5: The recording electrode next to a ruler (cm)

4.3.1. RECORDING ELECTRODE

The electrodes were custom made (AlphaOmega, Figure 5). The electrode consisted of 12 pins, six for placement in SI and six for placement in ACC, with 0.5 mm between the pins in each area. The pins were tungsten needles. The length of the pins differed so the electrode would fit into both areas.

4.3.2. SURGERY

Implantation of the electrode started with making an incision on the top of the head and removing the skin to the side. Two holes were drilled, one on each side of the midline. The right hole was used for the ground screw. From the left hole, a 6x4 mm hole was cut fitting the electrode. The dura was carefully retracted and the electrode placed so that the six pins in SI was at 1.5 to 2.0 mm posterior to Bregma, 1.0 to 3.0 mm lateral to the midline, and 1.4 mm ventral to the surface and the six pins in ACC at 0.5 to 2.0 mm anterior to Bregma, 0.5 to 1.0 mm lateral to the midline, and 2.7 mm ventral to the surface (Figure 6). The coordinates were based on Paxinos' rat atlas (Paxinos and Watson, 2007). The electrode was quickly inserted 6 mm further than the desired depth and then retracted to the correct depth. This procedure is a method to ensure that the pins are penetrating the correct layers of the cortex and so that there is no dimpling of the surface.

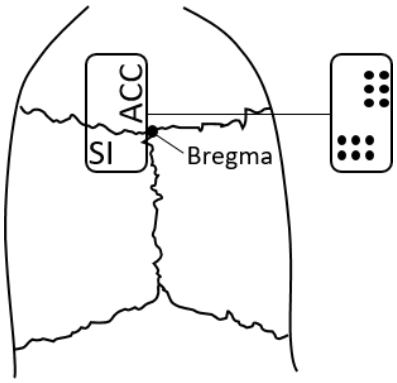

Figure 6: Illustration of the electrode placement in primary somatosensory cortex (SI) and anterior cingulate cortex (ACC)

4.4. PERIPHERAL ELECTRICAL STIMULATION

For two of the studies included in this thesis, electrically evoked potentials were analyzed. For this purpose, a custom made cuff stimulation electrode (Haugland, 1996) were made. The electrode consisted of two rings held in place by silicone (Figure 7, length: ~1 cm, diameter: 2 mm). The stimulation was controlled by two stimulus generators (STG2008 and SD9 stimulator). The stimulation consisted of mono-polar, 2 Hz, 100 μs pulse width, square waves and the amplitude was individualized.

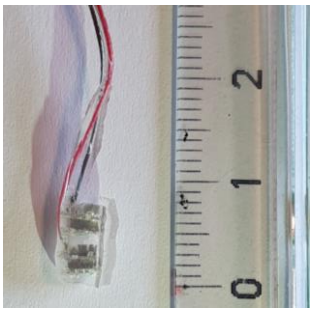

Figure 7: Cuff electrode for electrical stimulation next to a ruler (cm)

4.4.1. SURGERY

To implant the stimulation electrode, an incision was made through the skin above the m. biceps femoris on the left hind limb. The two parts of the muscle were separated using blunt scissors and the sciatic nerve and its branches carefully freed using cotton swabs. The recording electrode was placed around the nerve above the branches with a suture tied around to keep it in place. Also, sutures were placed around the nerve branches as preparation for the SNI.

4.4.2. NOXIOUS AND NON-NOXIOUS STIMULI

The intensity of the electrical stimulation was individualized to each rat based on the motor threshold. Two different non-noxious and one noxious stimuli were used in this thesis. The only difference between noxious and non-noxious stimuli was the intensity. Based on the study by Chang et al. (2001) showing a relation between electrical stimulation intensity and fiber type activation, 2 and 4 times motor threshold (low- and medium intensity) was used as non-noxious stimuli and 10 times motor threshold (high intensity) was used as noxious stimuli. The purpose of the different stimulus intensity was to recruit additional fiber types with higher stimulation.

4.5. RECORDING PARADIGM

The cortical activity was recorded every 30 min except for the first recording after the intervention, which was conducted as soon as possible after the induction of SNI (Figure 8). Each recording consisted of a period of 30-s resting-state followed by a 1-min period of evoked activity. The 30-s resting-state was a measure of spontaneous activity used in **Study III** but also as background activity that was subtracted from the evoked activity in **Study I** and **II** to limit the difference between subjects. The evoked activity was recorded during 2 Hz stimulation with either noxious or non-

noxious electrical stimulation. The stimuli were given in cycles with low, high, and medium intensity electrical stimuli in that order. One cycle was recorded before and three after SNI. For the control group, the surgery was the same except for the ligation and transection of the sciatic nerve that comprises the SNI. Instead, the control group was subjected to a 15-min wait as this was the approximate duration of the SNI intervention. This resulted in 12 recordings of cortical activity over approximately 5.5 hours, 4 recordings for each stimulation intensity.

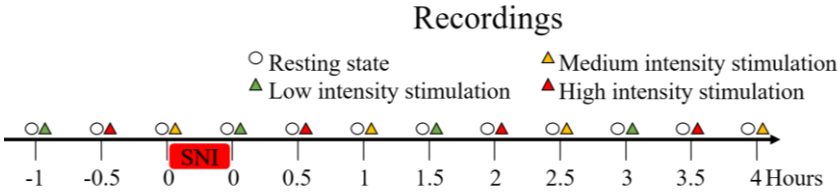

Figure 8: Timeline of the recordings relative to induction of the spared nerve injury (SNI).

4.6. **MULTI-UNIT SPIKE ANALYSIS**

The traditional approach for analysis of intracortical activity is by post-stimulus time histograms (Abeles, 1982). In this analysis, all spikes above a certain threshold are counted in bins and plotted as a histogram (Figure 9). In this way, time-locked spiking

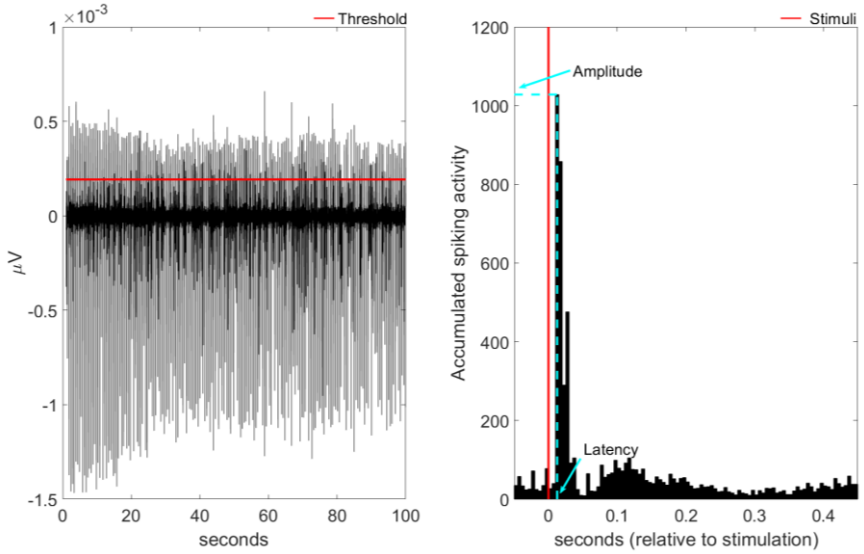

Figure 9: Illustration of multi-unit spike analysis. A threshold was used to calculate spikes from the raw signal (left) to create the PSTH (right). From the PSTH, the two features (amplitude and latency) were calculated.

activity relative to stimulation can be analyzed. To obtain the spiking activity, the raw signals were filtered between 800-3000 Hz (Figure 9, left). In this work, a PSTH analysis of the 50 ms before and 450 ms after stimuli with a 5 ms bin size was used. From the PSTH, the peak amplitude (accumulated spiking activity) and peak latency were analyzed (timing of the largest peak, Figure 9, right).

4.7. LOCAL FIELD POTENTIAL ANALYSIS

The LFP activity was analyzed through functional connectivity analysis. The raw signals were filtered between 1-200 Hz to obtain LFPs. Instead of using six electrodes from each area, one signal representing the whole area was calculated. The calculation was in two steps; first, a difference between the inner pin and the two outer pins resulted in two signals for each area, second, a difference between the two differential signals (double-differential) was calculated, resulting in one signal for each area (Figure 10). The double-differential signals were used in the following analysis. Four types of connectivity calculation were used in **Studies II** and **III**. These were Coherence, Correlation, Phase lag index (PLI), and Granger Prediction (GP).

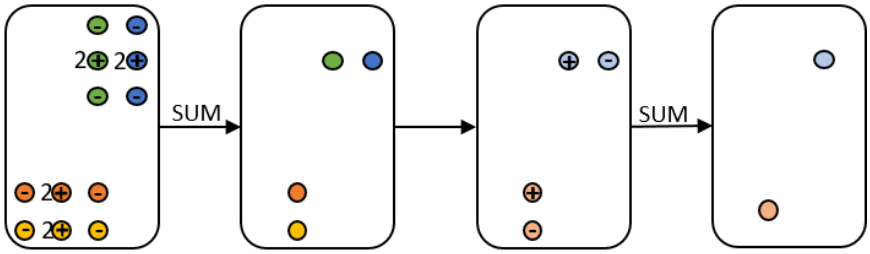

Figure 10: Calculation of the double-differential signals. The electrodes illustrated with the same color are summed in two steps, resulting in one signal representing ACC (blue) and on representing SI (orange).

Regardless of which connectivity calculation is used, the result is a number between 0 and 1, where connectivity close to 0 indicates weak connectivity and 1 indicates strong connectivity between areas. To calculate the Coherence, Correlation, and PLI, the pre-processed signals were further filtered into the classic EEG frequency bands (δ, θ, α, δ, γ, and high-γ) and the connectivity in each band was calculated. All three types of analysis are based on the analytic signal, which was calculated using the Hilbert-transform. Spearman's correlation was calculated using the Matlab function 'corr' and coherence as the absolute, squared cross-spectral density of SI and ACC, normalized with the power (spectral density) of the two areas (Cohen, 2014). PLI is based on the sign of the imaginary part of the cross-spectral density of the signals from SI and ACC (Cohen, 2014).

The GP was calculated using a customized Multi-variate granger causality Matlab toolbox (Seth, 2010; Barnett and Seth, 2014) providing the connectivity for a range

of frequencies within a specific area, in this case 1-100. To optimize the processing and avoid overfitting, data were down-sampled to 1 KHz. A 3rd order autoregression model was made, where the order was found using Bayes information criteria. The GP was calculated as a ratio between errors from a bivariate autoregression from both areas and univariate autoregression from each area (Cohen, 2014).

CHAPTER 5. SUMMARY OF MAIN RESULTS

This chapter summarizes the three studies introduced in Chapter 3: Outline of Ph.D. work (Tøttrup *et al.*, no date; L Tøttrup *et al.*, 2020; L. Tøttrup *et al.*, 2020). A brief overview of the main findings from these studies and how they relate to the three research questions is shown in Table 1.

5.1. SUMMARY STUDY I

The aim of **Study I** was to investigate the activation of SI and ACC to different intensities of electrical stimulation, and the modulation of amplitude and latency of spiking activity following a peripheral injury.

In **Study I**, electrically evoked cortical potentials (EECPs) were used to investigate spiking activity in SI and ACC before and after nerve injury. The multi-unit activity was recorded during three stimulation intensities (low, medium, and high) of electrical stimulation delivered through a cuff electrode on the sciatic nerve. Following a baseline recording of each stimulation intensity, the intervention group was subjected to the SNI model of neuropathic pain, while the control group was subjected to a 15 min wait (the approximate duration of the SNI procedure). Three recordings using each stimulation intensity was carried out post-intervention. A PSTH analysis of the spiking activity formed the basis of the signal analysis. Based on the PSTH, the amplitude and latency of the peak accumulated activity were compared across stimulation intensities and groups. Furthermore, these two features (amplitude and latency of the peak) were compared before and after SNI to investigate how the cortical response was modulated by SNI.

The results showed a higher amplitude of the response in ACC to high-intensity stimulation compared to medium- and low-intensity stimulation and in SI compared to medium-intensity stimulation. The peak response in SI was higher and faster than measured in ACC. Additionally, the results indicated that the response was modulated by SNI although this finding was not statistically significant. The response in SI to low-intensity stimulation in the intervention group increased after SNI to a level comparable to the response to high-intensity stimulation in the same group. In ACC, the peak latency of non-noxious stimuli decreased following SNI in the intervention group. This trend was not present in the control group.

These findings indicate that mechanisms similar to hyperalgesia and allodynia in humans can be investigated through cortical recording following a neuropathic pain model in rats. In support of the hypothesis that the response in both areas would

increase after injury and that the response in ACC would be slower than that in SI, the study showed increased activation of SI but not ACC. The findings also support the second part of the hypothesis as the response in ACC was slower than that in SI and even decreased after SNI.

5.2. SUMMARY STUDY 2

The aim of **Study II** was to investigate changes in directed connectivity from SI to ACC (and the other way) resulting from an intervention by SNI in the first hours.

In **Study II**, the interaction between SI and ACC during EECP was investigated before and after intervention with SNI. As in **Study I**, cortical activity was recorded from both areas during low-, medium-, and high-intensity stimulation. One cycle of recordings was conducted before and three after SNI in the intervention group and a 15-min wait in the control group. To investigate the SI-ACC interaction, both undirected and directed functional connectivity of LFPs were used. For the undirected connectivity, the phase-based measure PLI was calculated in six frequency bands in the 1-100 Hz range. The directed connectivity was calculated as GP in the same frequency range although across the frequencies without dividing into specific bands.

The results of the study showed an immediate decrease in PLI followed SNI in the intervention group. This was present across all frequency bands. Furthermore, the GP from SI to ACC in the high frequency range was increased hours after the intervention in the SNI group. There was no difference between the intervention and control group in directed connectivity from ACC to SI except for the last recording using low-intensity stimulation.

These findings indicate that there is an immediate reaction, possibly due to shock, to SNI seen as a decreased functional connectivity. Furthermore, the interaction between SI and ACC is more frequent (seen as more high-frequency GP) when the animal is subjected to SNI. The results of this study were in line with the hypothesis stating that the directed interaction from SI to ACC would be stronger than from ACC to SI and that this interaction would be stronger following injury, as a statistically significant increase in connectivity from SI to ACC after SNI was shown.

5.3. SUMMARY STUDY 3

The aim of **Study III** was to investigate how resting-state interactions between SI and ACC are altered by SNI, immediately and several hours after injury.

In **Study III**, the resting-state (or spontaneous) interaction between SI and ACC was investigated. Resting-state LFP activity was recorded before, immediately after, and three hours after SNI for the intervention group and similarly for the control group (where the SNI procedure was replaced by a 15-min wait period). The resting-state

interaction was quantified as coherence and correlation. Both the phase-based coherence and the temporal correlation was calculated in specific frequency bands. The six predefined frequency-bands used were the same as used in **Study II** and in most other LFP and EEG research. The functional connectivity from Coherence and Correlation was compared across recordings and groups.

The results showed a decreased correlation between SI and ACC in the intervention group immediately after SNI. The decrease was not specific to one frequency band. Three hours after injury, there seemed to be a decreased low-frequency and an increased high-frequency interaction, but these effects did not reach statistical significance. In general, the functional connectivity was increased in the control group in the first recording whereas it was increased in some frequency bands in the intervention group in the last recording three hours after injury.

The resting-state functional connectivity is affected by the SNI model but it is unclear exactly how and whether the resting-state activity is enough to identify cortical processes during a state of pain or injury. In support of the hypothesis that resting-state interactions were decreased immediately after SNI, the functional connectivity was decreased at the first recording but not three hours following injury.

Table 1: Summary of main findings from Study I-III related to each research question

	Study I: Modulation of SI and ACC response to noxious and non-noxious electrical stimuli after the spared nerve injury model of neuropathic pain	**Study II: Altered evoked low-frequency connectivity from SI to ACC following nerve injury in rats**	**Study III: Nerve injury decreases hyperacute resting-state connectivity between the anterior cingulate and primary somatosensory cortex in anesthetized rats**
Q1: To what extent does the SNI model result in a short-term (hours) increased response to non-noxious (allodynia-like) and noxious (hyperalgesia-like) stimuli?	• Shorter latency in ACC using non-noxious stim. • Larger peak-amplitude in SI using non-noxious stim.	• Increased SI→ ACC interaction hours after SNI to both noxious and non-noxious stim.	
Q2: How does functional changes occur in the first hours after intervention by the SNI model?		• decreased PLI immediately after SNI • Increased GP hours after SNI using all stim. intensities	•Decreased resting-state correlation
Q3: How does the evoked interaction between SI and ACC, and the resting-state interaction between SI and ACC after SNI differ?		• Increased evoked response but decreased resting-state response hours after SNI • Immediate decreased correlation and increased GP 1.5-4 hours after SNI	

CHAPTER 6. DISCUSSION

6.1. METHODOLOGICAL CONSIDERATIONS

6.1.1. ANESTHETIZED ANIMALS

One large issue with performing imaging or electrophysiology in animal studies is that the animals cannot be instructed to performed specific movements or not to move. When recording cortical activity in awake animals, several factors, such as movement artifacts and stress, may influence the recordings. On the other hand, when recording cortical activity in anesthetized animals, the level and type of anesthesia will influence the cortical signals. **Study I-III** is based on recordings from rats anesthetized with isoflurane. Isoflurane is known to suppress cortical spiking activity (Van Den Broek *et al.*, 2006; Aggarwal *et al.*, 2019), and connectivity (Jonckers *et al.*, 2014; Grandjean *et al.*, 2020; Xie *et al.*, 2020), but it has also been used during recordings of ERCPs (Rampil and Laster, 1992) similar to what is done in this thesis.

Most studies using anesthetized animals have a shorter time frame than the studies in this thesis. Thus, it is not known how the cortical activity is affected after being subjected to isoflurane for hours. One study using anesthetized monkeys with recordings for 4 hours showed depression of cortical signals (Li and Zhang, 2017) similar to short term studies.

The only way to increase the probability that the changes in cortical activity over time is actually due to the pain model is to use a control group. Thereby the changes seen in both groups are most likely due to anesthesia and possible further changes in the intervention group are likely due to the nerve injury. Additionally, in the statistical analysis in **Study II**, the anesthesia level was used as a covariate and found to not influence the results. It cannot, however, be ruled out that the isoflurane had an effect on the results, although it is unlikely with the precautions taken.

6.1.2. THE SNI MODEL TO STUDY NEUROPATHIC PAIN

Translation from animal research to humans has in many cases been problematic, especially in medical/pharmacological research, questioning the purpose of animal studies (Mogil, 2009; Mogil, Davis and Derbyshire, 2010). However, there are still many areas in which animal studies are considered important and relevant. It is important to notice that this work is not trying to explain or predict neuropathic pain but merely explore basic mechanisms in activation of and interactions between cortical groups of neurons. Similar to this study, many basic neurophysiological mechanisms are studied *in vitro* or *in vivo*.

Several animal models of neuropathic pain have been proposed and the models result in similar symptoms, such as thermal hyperalgesia and mechanical allodynia. In studies using these models, it is often presumed that the model is mimicking neuropathic pain without considering which model is used. In Zippo et al. (2016), however, two models of neuropathic pain and one model of inflammatory pain is used and compared, and it is shown that functional connectivity analysis provides distinguishable results for these models (Zippo *et al.*, 2016). Therefore, cortical processing depends on which models are used. This is an area requiring extensive additional research.

In **Study I-III**, the SNI model was used and cortical changes similar to those shown in other animal and human work was seen. The SNI model has been used extensively and validated through behavioral research (Baliki *et al.*, 2005; Pertin, Gosselin and Decosterd, 2012; Chang *et al.*, 2014). It may therefore be assumed that the model is in fact a representative model of pain though it can never be verified directly as the animals are not able to communicate their perception. As pain is a subjective phenomenon, the animal models will always result in reactions to the injury and not necessarily pain.

The SNI model is a model where two of three branches are completely transected and therefore mimicking injuries where a nerve is transected in humans. It is more common that the cause for neuropathic pain is diabetic neuropathy or postherpetic pain (Berge, 2011; Van Hecke *et al.*, 2014; Posso, Palmeira and Vieira, 2016). Other animal models besides the SNI model exist, where the injury is more similar to these conditions (Jakobsen and Lundbæk, 1976; Dalziel *et al.*, 2004; Fischer, Tan and Waxman, 2009). Furthermore, in human patients, the injury progresses over time and possibly heals or improves although this does not necessarily mean that the pain disappears. Reversible pain models, such as nerve crush models (Algora *et al.*, 1996; Decosterd and Woolf, 2000), could support investigation of this progression.

6.2. NEUROPHYSIOLOGICAL MECHANISMS

6.2.1. CORTICAL RESPONSE TO NOXIOUS STIMULI

In **Study I**, it was shown that the cortical response in SI and ACC increased with higher intensity electrical stimulation. Furthermore, the response in SI increased after SNI in **Study I and II**. The increase in cortical processing of both noxious and non-noxious stimuli after SNI in the intervention group may be an indication of allodynia- and hyperalgesia-like responses, similar to those seen in neuropathic pain patients (Scholz *et al.*, 2019). Allodynia and hyperalgesia-like mechanisms have been shown previously in rats in both behavior (Baliki *et al.*, 2005; M. N. Baliki *et al.*, 2014; Chang *et al.*, 2017) and cortical activation (Chao, Chen and Yen, 2018).

The mechanisms underlying these increases are probably too fast to be the formation of a new connection and it is likely that central sensitization is beginning to occur and that previously silent synapses are activated.

6.2.2. FUNCTIONAL CHANGES AFTER SNI

In a short time interval, such as the first recordings after injury in this thesis, the effect of the shock of nerve denervation may be the cause of the initial decrease in resting-state connectivity found in **Study II** and **III**. Furthermore, studies on evoked responses following animal models of pain found increased cortical responses after intervention showed as increased firing rate (Singh *et al.*, 2020) or LFP power (LeBlanc *et al.*, 2014, 2016; Zhang *et al.*, 2018; Xiao *et al.*, 2019). In **Study I**, a non-statistically significant increased response was found. One reservation that should be made is that the nerve that is being stimulated is the same as the one being subjected to SNI. Therefore, a decreased response to stimuli could be expected as there are fewer nerve fibers to stimulate.

The sustained increased evoked connectivity in the intervention group found in **Study II** could be linked to cortical plasticity. It is, however, noticeable that with only cortical recordings, peripheral or spinal changes cannot be ruled out. Several studies with a longer time frame (days/weeks) find similar increases in evoked responses after neuropathic (Zippo *et al.*, 2015; Shih *et al.*, 2019) or inflammatory pain (Tan *et al.*, 2019; Guo *et al.*, 2020) in electrophysiological recordings but these also only include cortical recordings.

6.2.3. EVOKED AND RESTING-STATE CONNECTIVITY

In **Study II** and **III**, it was shown that SNI results in an initial decrease in resting-state functional connectivity and an increased evoked functional connectivity hours after injury. Several previous studies show similar findings, both in regards to a decrease in resting-state functional connectivity days after a model of neuropathic pain (LeBlanc *et al.*, 2014) which is increased weeks later (Zippo *et al.*, 2015; LeBlanc *et al.*, 2016), and increased evoked connectivity days after a model of neuropathic pain (Zippo *et al.*, 2015; Shih *et al.*, 2019) and hours inflammatory pain (Tan *et al.*, 2019; Guo *et al.*, 2020).

In most previous literature, the findings for resting-state connectivity are opposite to those of evoked connectivity in the hours or days after an intervention. Whereas findings from electrophysiological recordings show a decreased resting-state connectivity, the evoked-connectivity is increased until weeks after an injury where these findings are reversed (Table 2). One fMRI study did find an immediate (minutes) increased resting-state connectivity (Chao, Chen and Yen, 2018). It is important to point out that these studies are not necessarily investigating the functional connectivity between SI and ACC but just connectivity between areas traditionally

related to pain processing. It is also evident that there is not extensive research in this area.

The increase in evoked connectivity and decrease in resting-state connectivity may be an implication of the functional changes not necessarily being cortical. If the changes in the neural system e.g. were due to central sensitization in the spinal cord or other subcortical functions, it is likely that only the evoked connectivity would reflect this.

*Table 2: Previous finding of resting-state and evoked connectivity in neuropathic, and other pain models. *Only overall significant but not for specific frequency bands.*

Neuropathic pain models					
		Resting-state		Evoked potentials	
Hours	Low freq (δ, θ)	↓	**Study III***		
	Med Freq (α,β)	↓	**Study III***		
	High freq (γ+)	NS	**Study III***	↑	**Study II**
	fMRI	↑	(Chao, Chen and Yen, 2018)		
Days	Low freq (δ, θ)	↓	(LeBlanc *et al.*, 2014)		
	Med Freq (α,β)	↓	(LeBlanc *et al.*, 2014)		
	High freq (γ+)	↓	(LeBlanc *et al.*, 2014)		
	fMRI	NS/ ↑	(Marwan N. Baliki *et al.*, 2014)/(Chao, Chen and Yen, 2018)		
Weeks	Low freq (δ, θ)	↑	(Zippo *et al.*, 2015; LeBlanc *et al.*, 2016)	↓	(Zippo *et al.*, 2015)
	Med Freq (α,β)	↑	(Zippo *et al.*, 2015; LeBlanc *et al.*, 2016)	↓	(Zippo *et al.*, 2015; Shih *et al.*, 2019)
	High freq (γ+)	↑	(Zippo *et al.*, 2015)	↓	(Zippo *et al.*, 2015; Shih *et al.*, 2019)
	fMRI	NS	(M. N. Baliki *et al.*, 2014)		

One important aspect of comparison of resting-state and evoked functional connectivity is that in **Study I** and **II**, different methods of calculating connectivity were used, which limits the comparison. The results are, however, consistent with previous findings, although only Zippo et al. (2015) used the same connectivity methods for the analysis of both resting-state and evoked connectivity and found opposite changes in evoked and resting-state connectivity weeks after neuropathic injury. The methods used in **Study III** were coherence and correlation, which are the simplest and most frequently used in other functional connectivity studies and thereby easier to compare to previous research. In **Study II**, PLI was used to investigate undirected connectivity, as it is more robust to field spread/volume conduction. There is, however, always a probability that connectivity between two areas could be resulting from a third area connected to both (common input problem) (Cohen, 2014). Additionally, GP was used to investigate directed connectivity between SI and ACC. This method is more complex and difficult to calculate and only a few previous studies in this research field have used GP. In Guo et al. (2020), it was used to investigate evoked connectivity following a model of inflammatory pain (Guo *et al.*, 2020) with results similar to the results in **Study II**.

6.3. IMPACT OF THE PHD WORK

In the previous literature of cortical functional changes following animal models of neuropathic pain, the first minutes and hours after the intervention is largely uninvestigated. **Study I-III** demonstrated that functional changes do seem to occur in this period. With these studies, additional knowledge to the previous knowledge base has been added (see green circles in Figure 11).

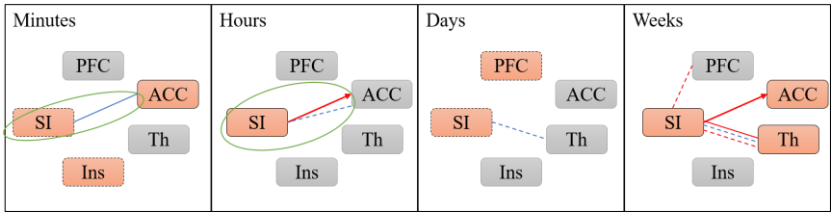

Figure 11: Additions to the current knowledge from previous literature (see state-of-the-art). The green circles show the contributions from the three studies which this thesis is based on. Red boxes/lines indicate increase and blue lines indicate a decrease of activity (boxes) and functional connectivity (lines). Grey boxes indicates unreported or uninvestigated areas and connections. Dotted lines indicate resting-state studies and full lines, evoked potentials. Ins: Insular cortex, Th: Thalamus

The work in this thesis has begun to open up the black box relating to understanding the neurophysiological mechanisms in the minutes and hours following spared nerve injury (i.e. a model of neuropathic pain). The three studies, and in particular **Study II**, show that cortical changes do occur in the short time span that is the hours after injury. This means that the cortical plasticity may be affected long before previously

believed. Understanding the timeline of cortical plasticity after an injury is necessary to understand chronification and, in time, develop a treatment.

The most important next step in this line of research is to use the same protocol in semi-chronic studies. Semi-chronic in this case would be days or possibly up to one week after the intervention. With this approach, would be easier to compare and validate the results with previous studies. Furthermore, studies enabling comparisons of both models of chronic pain but also the evoked responses to different types of noxious and non-noxious stimuli are necessary. Most studies are using only one model and one stimulus modality, which makes comparison difficult across studies. Due to both ethical reasons and the large expenses related to animal studies, most research is conducted in relatively small sample sizes, not allowing comparison between models of pain, stimulus modalities to evoke pain or species of animals used as it would require a large sample size to reach satisfying statistical power.

6.4. CONCLUSIONS

No single cortical area or combination of cortical areas have been found to have specific pain activation and it can be hypothesized that the specificity to pain is not found in the activation *per se*, and it is therefore relevant to investigate the interactions. This thesis aimed to investigate the cortical response in an animal model of neuropathic pain in the first hours following injury.

To address this aim, three research questions were formulated and three studies conducted:

Q1. To what extent does the SNI model result in a short-term (hours) increased response to non-noxious (allodynia-like) and noxious (hyperalgesia-like) stimuli?

In **Study I** and **II**, it was shown that the activation of SI and interaction between SI and ACC increases following a peripheral nerve injury. This increase was both to low-intensity non-noxious electrical stimulation and to high-intensity noxious electrical stimulation which respectively indicate allodynia-like and hyperalgesia-like mechanisms.

Q2. How does functional changes occur in the first hours after intervention by the SNI model?

In **Study II** and **III**, the functional connectivity between SI and ACC was altered in the first hours after SNI. An increased evoked connectivity three hours after SNI was shown in **Study II** and a decreased resting-state connectivity was shown in **Study III**.

Q3. How does the evoked interaction and the resting-state interaction between SI and ACC differ after SNI?

Comparison of the results from **Study II** and **Study III** showed that the changes in evoked and resting-state interactions were opposite. While the SNI resulted in an increased evoked functional connectivity, it also resulted in a decreased resting-state functional connectivity. Furthermore, the decreased resting-state functional connectivity occurred immediately after SNI while the increase in evoked functional connectivity did not reach statistical significance until hours after injury.

In summary, a significant change in SI-ACC interaction was found within hours after the SNI model in **Study I-III**. The immediate effect of SNI is a decreased interaction between SI and ACC, which is followed by increased activation and interaction. The immediate response may be due to a shock from the injury, whereas the changes in the hours after injury are possibly caused by central sensitization or cortical neuroplasticity.

TABLE OF FIGURES

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