Interactions with pain-related systems - Towards new electrical treatments for chronic pain

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Interactions with pain-related systems

Towards new electrical treatments for chronic pain

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Abstract

Background. Persistent intolerable pain is still an unsolved issue with a huge socioeconomic impact. To develop appropriate treatments, it is crucial to understand the complex mechanisms underlying pain and how they change during sustained pain stimuli or during pathological conditions. In particular, it is essential to clarify how the endogenous analgesic centres which are present in the brain, such as periaqueductal grey (PAG) matter and dorsal raphe nuclei (DRN) in the brainstem, modulate pain by interfering with the nociceptive information. Deep brain stimulation of these areas can elicit potent analgesia. However, it has not been possible to exploit its full analgesic potential due to the insufficient stimulation specificity of the state-of-the-art probes.

Aim. To address this challenge, we developed and implanted in rodents, a novel probe for high-definition brain stimulation (HDBS) based on the spread in 3D of ultra-flexible microelectrodes in PAG/DRN. The probe comprised 16 ultra-flexible microelectrodes embedded in a gelatine needle-like probe. The main aim was to elucidate whether stimulation of individually selected microelectrodes can selectively activate the anti-nociceptive pathways without activating networks that provoke side effects.

Methodology. The selection of an appropriate microelectrode subset and stimulation intensity was done by monitoring the withdrawal reflexes elicited by CO_2 laser stimuli and by simultaneous behavioural observations in awake freely moving animals. To evaluate the effect of HDBS in PAG/DRN on the nociceptive pathways related to pain perception, microelectrode recordings were made in cortical areas known to be involved in the sensory-discriminative and affective aspects of pain. Clinically relevant aspects such as potency, specificity, sustainability, reliability of HDBS as well as efficacy in conditions with hypersensitivity to nociceptive stimuli (hyperalgesia) were assessed by recording nociceptive-evoked cortical responses, withdrawal reflexes, gait and normal behaviours. To assess the presence of side effects, HDBS effect on intracortical spontaneous activity, brain states (ECoGs), behaviour in an open field, and the tactile input to cortex was also investigated. The tissue reactions to the implanted stimulation probe and the probe placement were evaluated using immunohistochemistry. In addition, we investigated whether tissue reactions related to probe implantation can be mitigated by incorporating PLGA-nanoparticles loaded with an anti-inflammatory drug, minocycline, and embedded into a gelatine vehicle.

Results. For all the animals with verified placement within or nearby PAG/DRN, it was possible to individually select a microelectrode subset and stimulation intensity, which strongly inhibit nociceptive-evoked withdrawal reflexes without noticeable side effects. The selected microelectrode combinations also reduced nociceptive-evoked cortical responses (related to both discriminative and affective pain) in normal conditions and during hyperalgesia. The HDBS-induced analgesia could be sustained for at least 4 hours and did not provoke significant side effects on behaviour, spontaneous activity, and brain states and it had a minor effect on the tactile afferent pathway to the cortex. Histological analysis showed minimal tissue reactions and neuronal death around the stimulation implant. Minocycline containing PLGA nanoparticles significantly reduced glial reactions without signs of toxicity.

Conclusions. These results show that granular and high-resolution PAG/DRN stimulation enables potent, specific, safe, and durable analgesia by blocking the nociceptive-evoked motor, sensory and affective responses without significant activation of pathways provoking adverse side effects. Therefore, HDBS in PAG/DRN holds great promise as an efficient treatment of intractable chronic pain disorders.

Keywords
High-definition deep brain stimulation, Periaqueductal gray matter, Pain systems, Pain models

Classification system and/or index terms (if any)

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To my grandmother Ida,
who wanted to study the brain

Notes on the cover painting

Title: Tortuosity

Artist: Maurizio Fava (neuroscientist, artist, and philosopher)

Technique: Paint on canvas (laboratory pipettes were used to paint the labyrinths)

Year: 2020

Description: The labyrinth of Daedalus (from labrys*, double axe, solar and royal symbol) was designed by the Cretan king Minos to protect the population from the monstrous Minotaur, half man and half bull. Borges describes his obsessive dream as follows: "A small, clean labyrinth, in the centre of which there is an amphora that I almost touched with my hands, that I saw with my own eyes, but the streets were so twisted, so confused, that one thing seemed clear to me: I would have died before I got there ". Today there is no Theseus, there is no Arianne. There is only a labyrinth. And we are the Minotaur: cloned, mechanized. Fused with machines and artificial technologies. Our bellow rises desperately to a merciless sky. Ice cream is enough to console us, to compensate us. We asked for it. The smurf-blue and greenish colour, as if squeezed out of a tube of paint, has the vague scent and flavour of an anise and pistachio top.*

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Popular scientific summary

Intense or light, short or persistent, all of us have experienced pain. These experiences have a common feature: they were most likely unpleasant. That is because, by teaching and motivating us to avoid dangers, pain is essential for our survival. But imagine having a strong constant pain somewhere in your body: could you cope to perform work, or manage to sleep? What if it would last for years? How much would it affect your life? Unfortunately, many people suffer from chronic pain conditions, and efficient treatments are often lacking, or they cause severe side effects such as sedation.

That is what gives the motivation of this thesis, trying to solve the problem with… electricity! The brain is populated by neurons which talk to each other by sending electrical signals. In this way, they control every function of our body and consciousness, including pain. Here is the key: we can modify the pain messages sent by neurons delivering small electrical pulses in the right place and in the right way.

The method of interfering with the electrical activity of neurons has been known for several decades. In the 1950s it was discovered that electrical stimulation of a specific area called "periaqueductal grey matter" which is located deep in the central core of rat brains, could provoke complete shut-down of pain. However, the success in curing or treating pain conditions reliably and efficiently remained a challenge since the electrodes used for stimulation, delivered too large electrical fields which were activating not only the neurons involved in pain but also neurons involved in other functions, thus provoking unpleasant side effects.

The approach introduced in this thesis was to use a new family of electrodes allowing for a very controlled and high-resolution stimulation compared to any previously used electrodes. This new technique for brain stimulation is based on the insertion of several electrodes with microscopic dimensions in the periaqueductal grey matter of rodents. These microelectrodes could be activated, inactivated, and regulated singularly. By selecting the right ones, it was possible to obtain a very strong blockage of pain without provoking side effects.

In parallel to the stimulation technology, another challenge of the pain field was faced in this thesis: how is it possible to measure pain in rodents? That is a difficult task since it is not possible to ask the animals how they feel. So, scientists study the animals´ behaviour or their pain-triggered withdrawals. However, these methods

can lead to several misinterpretations. Instead, we employed an alternative and unbiased method to measure pain.

When we receive a pain stimulus, our neurons transform the input into a signal that is possible to detect from specific areas in the brain involved in pain perception. That signal is what was used in this thesis to detect pain in animals. A pain stimulus was used to produce a pain signal which was recorded with electrodes implanted in two brain areas called the “somatosensory cortex” and the “cingulate cortex”, both involved in different aspects of the pain experience. This pain signal was almost completely gone when we stimulated the periaqueductal grey matter, meaning that the pain perception was inhibited.

While the pain measurement approach in animals gives more reliability to the results obtained in preclinical animal research, the innovation of the neuron stimulation technique increases the probability of successful pain treatment without side effects. Together, these innovative techniques raise hopes for better treatments for all people who suffer from chronic pain disorders in the near future.

Populärvetenskaplig sammanfattning

Intensiv eller lätt, kort eller ihållande, alla av oss har upplevt smärta. Dessa upplevelser har ett gemensamt drag, de var alla med största sannolikhet obehagliga. Det beror på att smärtan är avgörande för vår överlevnad, genom att lära och motivera oss att undvika faror. Men tänk dig att ha en stark konstant smärta någonstans i kroppen, skulle du klara av att utföra arbete eller att sova? Tänk om det skulle hålla i sig i flera år? Hur mycket skulle det påverka ditt liv? Tyvärr lider många människor av kroniska smärttillstånd och effektiva behandlingar saknas ofta, eller så orsakar de allvarliga biverkningar som allvarlig trötthet och dåsighet.

Det är denna bakgrund som ger motivationen till den här avhandlingen där vi försöker bota smärta med hjälp av elektricitet!

Hjärnan är uppbyggd av nervceller som pratar med varandra genom att skicka elektriska signaler. På detta sätt kontrollerar de varje funktion av vår kropp och medvetande, inklusive smärta. Det är här vi har nyckeln, vi kan modifiera smärtmeddelanden som skickas elektriskt av nervceller genom att levererasmå elektriska pulser på rätt plats och på rätt sätt.

Metoden att störa den elektriska aktiviteten hos nervceller har varit känd i flera decennier. På 1950-talet upptäcktes att elektrisk stimulering av ett specifikt område som kallas "periakveduktal grå massa" som ligger djupt inne i råtthjärnans hjärna, kunde leda till fullständig avstängning av smärta. Möjligheten att bota eller behandla smärttillstånd på ett tillförlitligt och effektivt sätt förblev dock en utmaning eftersom elektroderna som användes för stimulering, levererade för stora elektriska fält som aktiverade inte bara nervcellerna som var involverade i smärta utan även nervcellerna involverade i andra funktioner, vilket provocerade fram obehagliga sidoeffekter.

Innovationen som introduceras i denna avhandling består i en ny typ av elektroder som möjliggör en mycket kontrollerad och högupplöst stimulering jämfört med alla tidigare använda elektroder. Denna nya teknik för hjärnstimulering är baserad på införandet av flera elektroder med mikroskopiska dimensioner i den Periakveduktal grå massan hos gnagare. Dessa mikroelektroder kan aktiveras, inaktiveras och regleras var för sig. Genom att välja rätt elektrod var det möjligt att aktivera endast nervcellerna som är involverade i smärthämning och på så sätt undvika biverkningar.

För att kunna utvärdera stimuleringstekniken behövdes en annan utmaning för smärtområdet adresseras i denna avhandling: hur är det möjligt att mäta smärta hos djur? Det är en svår uppgift eftersom det inte går att fråga djuren hur de mår. Så traditionellt studerar forskare djurens beteende eller deras smärtutlösta reflexer. Dessa metoder kan dock leda till flera feltolkningar. Istället använde vi en alternativ och opartisk metod för att mäta smärta.

När vi får en smärtstimulans omvandlar våra neuroner stimulit till en signal som är möjlig att upptäcka från de specifika områden i hjärnan som är involverade i smärtuppfattning. Den signalen är vad som användes i denna avhandling för att upptäcka smärta hos djur. Ett smärtstimulus användes för att producera en smärtsignal som registrerades med elektroder implanterade i två för smärta viktiga hjärnområden som kallas "somatosensoriska kortex" och " gyrus cinguli ", båda involverade i olika aspekter av smärtupplevelsen. Denna smärtsignal var nästan helt borta när vi stimulerade den periakveduktala grå massan, vilket innebär att smärtuppfattningen hämmades.

Eftersom vår smärtmätningsmetod ger mer tillförlitliga resultat från djurförsök än som erhållits i traditionell preklinisk forskning så ökar möjligheterna att fin-justera nervcells-stimuleringstekniken för en framgångsrik smärtbehandling utan biverkningar. Tillsammans väcker dessa innovativa tekniker en förhoppningar om bättre behandlingar för alla människor som lider av kroniska smärtsjukdomar inom en snar framtid.

Original papers included in the thesis

I. **Forni Matilde**, Thorbergsson Palmi Thor, Thelin Jonas, Schouenborg Jens. *3D microelectrode cluster and stimulation paradigm yield powerful analgesia without noticeable adverse effects*.
Science Advances. 7(41), eabj2847. 2021 October 8.

II. **Forni Matilde**, Thorbergsson Palmi Thor, Gällentoft Lina, Thelin Jonas, Schouenborg Jens.
Sustained and powerful analgesia with minimal behavioral and brain state side effects induced by high-definition brainstem stimulation in rats.
Manuscript submitted for publication.

III. **Forni Matilde**, Thorbergsson Palmi Thor, Gällentoft Lina, Retelund Alle, Thelin Jonas, Schouenborg Jens.
Abolished nociceptive signalling in cortical regions involved in sensory and affective aspects of pain by high-definition brainstem stimulation.
Manuscript.

IV. Holmkvist Dontsios Alexander, Agorelius Johan, **Forni Matilde**, Nilsson Ulf, Linsmeier Eriksson Linsmeier, Schouenborg Jens.
Local delivery of minocycline-loaded PLGA nanoparticles from gelatin-coated neural implants attenuates acute brain tissue responses in mice.
Journal of Nanobiotechnology. 18(1):27. 2020 February 5.

Abbreviations

-A	Inverted Amplitude
ACC	Anterior Cingulate Cortex
AP	Anteroposterior
Avg. Z-score	Averaged z score
Avg. speed	Averaged speed
CD68	Cluster of Differentiation 68
CO_2	Carbon Dioxide
CX3CR-1	CX3C Motif Chemokine Receptor 1
DAPI	4′,6-diamidino-2-phenylindole
DBS	Deep Brain Stimulation
DRN	Dorsal Raphe Nuclei
DV	Dorsoventral
ECoG	Electrocorticography
ED1	Ectodysplasin A
FP	Field Potential
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
HDBS	High-Definition Brain Stimulation
I_{max}	Maximal Current
IOI	Interval of Interest
ML	Mediolateral
MUA	Multiunit Activity
NeuN	Neuronal Nuclei

N_2O	Nitrous Oxide
n.s.	Non-significant
PAG	Periaqueductal Grey
PLGA	Poly(Lactic-co-Glycolic Acid)
PSD	Power Spectral Density
O_2	Oxygen
ROI	Region Of Interest
p_{moving}	Probability of moving
PS	Post-surgery
S1	Primary Somatosensory Cortex
REM	Rapid Eye Movement
rTMS	Repetitive Transcranial Magnetic Stimulation
TENS	Transcutaneous Electrical Nerve Stimulation
UVB	Ultraviolet B

Introduction

The relevance of this thesis

Persistent pain has a major societal impact worldwide due to the impairment of the quality of life for the patient, the healthcare burden, and its high financial burden on society. It has been estimated that approximately 20% of the global population is affected by chronic pain [1]. In Sweden, the socioeconomic cost of chronic pain-related diseases has been estimated to be around 10% of the gross domestic product [2]. Chronic pain is also more difficult to relieve than acute pain and often associated with parallel issues such as sleep disturbances, depression, and social problems [2, 3]. In addition, patients with intractable pain conditions are often affected by mental illnesses which can possibly increase the risk of suicide [4, 5].

Unfortunately, current analgesic therapies are often ineffective, unreliable or cause severe side effects (such as sedation, gastric problems, or addiction). There are several reasons related to this: i) the limited knowledge about the complex pain system; ii) the wide aetiology underlying pain conditions; iii) the difficulty to measure and modulate pain since it is a subjective sensation; iv) the lack of appropriate and valid animal models to study pain.

Because of the immense impact of chronic pain on our society, there is an urgent need to understand the biological mechanisms of pain and how to modulate them.

The physiology of pain

General overview

Most of us have most likely touched a hot frying pan without heat protection, and while retracting our hand, realized that it was a mistake. Even though the experience was probably unpleasant, it reveals how essential is pain for the protection of our bodies. The unpleasant feeling of the experience is useful to motivate us to avoid such situations, to learn from them as well as to prevent them. However, the sensation of discomfort and the reflection on the experience come after we have already retracted the hand. A withdrawal reflex is quickly activated to protect the

body from damaging stimuli before it has caused cellular and tissue damage. Thus, when we realize what happened, our arm is already far from the frying pan.

Pain is essential for survival and people with congenital pain insensitivity have a lower life expectancy since they don´t feel motivated to adapt their behaviour to avoid injuries [6]. The International Association for the Studies of Pain defines pain as "*an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage*" [7]. This definition highlights two important concepts. The first is that a painful experience includes two aspects: the actual painful sensation (the discriminative pain, which contains conscious information about the location and intensity) and the negative affective sensation we usually attribute to that. These two components are often correlated, but they can be dissociated in certain conditions since they are based on different neural substrates and processes [8-10]. The second is that pain does not require damage to be "real". This often happens in non-physiological pain conditions where pain can be felt without an external stimulus.

But what is the neural substrate of pain? Firstly, it is important to differentiate *pain*, which is the feeling of distress, from *nociception*, which is the underlying physiological process, which leads to the neural encoding of a noxious stimulus. The result of this neural processing is the subjective conscious sensation of discomfort we call "pain". In addition, nociceptive pathways also activate pre-cognitive behavioural responses such as withdrawal reflexes and autonomic responses.

The nociceptive afferent pathways start from peripheral nociceptors which are free-ending nerve fibres. They can be activated by mechanical, heat and chemical noxious stimuli and belong to either relatively rapidly conducting Aδ fibres or slowly conducting C fibres [11]. The different nomenclature of the fibres corresponds to different diameters and myelinisation of fibres, which correlates to different transmission speeds. These fibres, also called first-order neurons, enter through the dorsal horn of the spinal cord where they synaptically connect with second-order neurons (figure 1a). In most cases, the second-order neurons send axons that cross the midline and ascend to higher structures through the spinothalamic or spinoreticular tracts [11]. Thalamus is a key "relay centre" in the transmission of nociceptive signals where third-order neurons project to the primary and secondary somatosensory cortices, the insula, the anterior cingulate cortex and the prefrontal cortex for both sensory/discriminative and affective/emotional processing of pain (figure 1a) [12, 13]. However, other structures seem to be involved in some aspects of nociception, such as the hippocampal complex, the amygdala, the red nucleus, and the brainstem [14]. In parallel, polysynaptic spinal withdrawal reflex modules rapidly coordinate the muscle activity of a limb causing its withdrawal from the potential damage [15].

Probably most of us have noticed that injuries, normally causing pain, may not always be felt when they happen, maybe because we were in a dangerous situation, doing sport, or because we were very concentrated on something else. This can happen since pain perception can be modulated depending on the context. The nociceptive system also involves descending pathways engaged in pain modulation. Some of the most powerful centres are the periaqueductal grey (PAG) and the nucleus raphe magnus in the brainstem (figure 1b). These centres are activated in specific situations (such as exposure to threats or stress) and modulate the nociceptive afferent pathways at the level of the spinal cord [16].

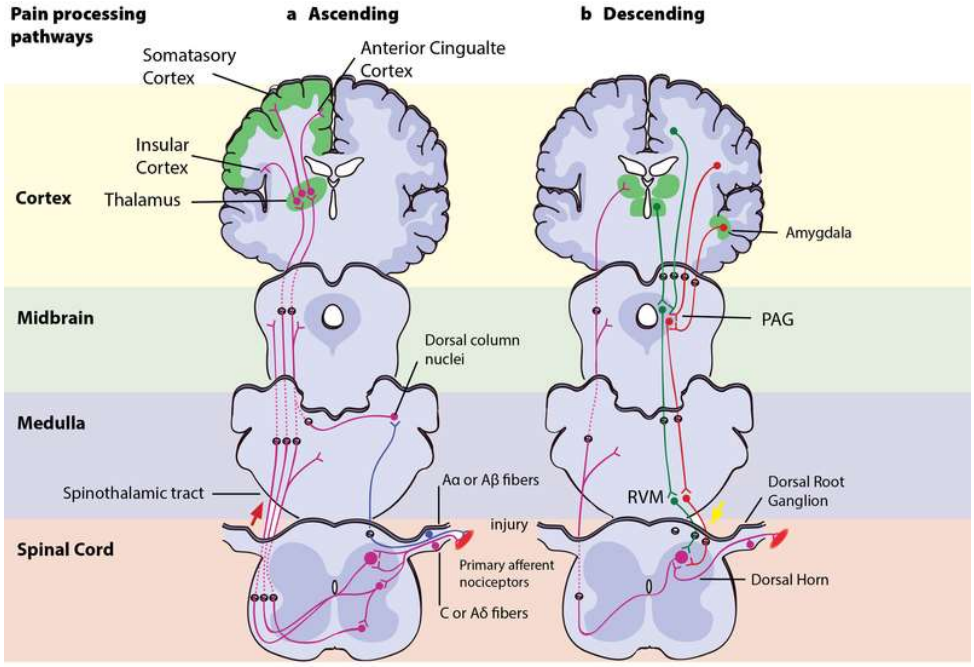

Figure 1. Schematic of the pain processing pathways.
Simplified overview of ascending (**a**) and descending (**b**) pain-related pathways in the central nervous system. Reproduced with permission from Creative Commons´ open access policy from [17]. PAG, periaqueductal grey; RMV, rostal ventromedial medulla.

The interplay of all these structures and neuronal pathways with different roles and biological composition creates the complex puzzle of an apparently diffuse pain system whose overall activity results in pain perception. This widespread neural network integrates sensory-discriminative, motivational-affective, and cognitive-evaluative components of the pain experience as well as pre-cognitive physiological and behavioural responses [18-20].

In front of such an intricate organization, of which very little is known, it is not surprising that the goal of understanding and controlling pain is still a huge challenge which awaits to be solved.

Pain integrative centres

Primary somatosensory cortex

The primary somatosensory cortex (S1) in humans is defined as the cerebral area in the postcentral gyrus and includes the Brodmann areas 3, 1, and 2. It receives somatic information regarding e.g. touch, pressure, temperature, vibration, nociception, and proprioception from the musculoskeletal system, and it is also involved in the integration and processing of sensory and motor information [21, 22].

S1 was first described in 1937 in an article entitled "*Somatic motor and sensory representation in the cerebral cortex of man as studied by electrical stimulation*" by Wilder Penfield and Edwin Boldrey [23]. They performed surface electrical stimulation of cortical areas and collected data based on motor outputs or on the description of the patient sensations during brain surgeries of an extensive sample of awake patients. In this way, they identified the somatosensory area, which was receiving massive sensory input, and the motor area, which was provoking motor actions when stimulated. They drew for the first time the *Homunculus* which is a collective map of the sensorial and motor representation in the brain.

S1 receives nociceptive information from the thalamus of sensory-discriminative pain and is usually considered the first level of pain consciousness [24]. It has been shown that the signals received by the S1 contain information about both location and intensity of pain [24, 25]. This information can be measured using recordings of field potential (FP) signals in S1 and have features resembling the ones measured in awake freely moving rats [26, 27].

Anterior cingulate cortex

The anterior cingulate cortex (ACC) lies in the front portion of the cingulate cortex which surrounds the frontal part of the corpus callosum. It is positioned in between and has connections with both the limbic system (involved in emotional functions) and the prefrontal cortex (involved in several cognitive functions). Due to its location, it is believed to play an essential role in the integration of cognitive and emotional information and is involved in several complex higher cognitive functions, such as decision-making, empathy, emotion, impulse control, attention, reward expectancy, error detection, and pain [28, 29].

Most of the research on ACC involvement in pain has been performed just recently because the earlier studies on pain used mainly withdrawal reflexes as a pain-

assessment method, which does not give information about the unpleasant aspect of an acute or persistent pain condition.

Through the use of novel models and methods for both animal and human studies, it was possible to demonstrate that ACC has a major role in the affective-emotional component of pain [8, 30, 31]. This area, which receives and processes nociceptive information from cortical, thalamic, hypothalamic, and brainstem projections, seems to be necessary also to develop a motivational force to avoid a painful emotion [32]. Observations supporting this notion include studies in which ACC lesions were found to correlate to a decrease in affective pain experiences in humans [33] and a disability to learn noxious stimulus avoidance in animals [34]. ACC structural cortical layer changes have also been observed during chronic pain in rodents [35]. Recent findings also showed that there are a population of mirror neurons within the ACC which are activated when witnessing a painful experience in another individual [36].

The periaqueductal grey and dorsal raphe nuclei

The PAG matter is a midbrain area located around the cerebral aqueduct which is composed of cell nuclei involved in a variety of autonomic and behavioural functions, such as cardiovascular, respiratory, motor, and pain control, but also thermoregulation, bladder muscles control, vocalization, rapid eye movement (REM) sleep, sexual and maternal behaviour [16, 37, 38].

PAG has been subdivided into four different columns (dorsomedial, dorsolateral, lateral and ventrolateral), based on their cyto/chemo-architecture and functional connectivity. The columns run in parallel to the cerebral aqueduct, through which the PAG coordinates responses to threats and motivated behaviour [16, 39, 40].

The dorsolateral and lateral columns are thought to be associated with coordinating sensorimotor and autonomic behavioural responses, such as hypertension, increased heart and breath rate, and analgesia, during a flight or confrontational defence situation. The ventrolateral column instead activates different strategies during stressful situations where behavioural responses such as quiescence, hypotension, decreased heart and breath rate, and analgesia are activated [39, 41].

The dorsal raphe nucleus (DRN) is part of the rostral ventromedial medulla and is located below the cerebral aqueduct and bordering the ventrolateral PAG. It is the major serotoninergic nucleus containing approximately one-third of all the serotoninergic neurons present in the brain [42]. The DRN is involved in orchestrating several functions among which sleep/wake cycle [43], social interactions [44, 45], reward [46, 47] and pain regulation [48, 49].

There are several studies, which indicate that PAG/DRN is a key component in the top-down pain modulation network [50-55]. Its action is coordinated by the interplay of the different PAG columns activation which indirectly connects to the

dorsal horn via the dorsal ventromedial medulla modulating the nociceptive signal transmission (Figure 1b) [53, 55-60]. PAG also exerts its anti-nociceptive action with projections to noradrenergic brain stem nuclei, which, in turn, connect to the dorsal horn [61]. A few studies have reported that the dorsolateral PAG activates sympathetic reactions during escape or fight situations and short-lasting non-opioid analgesia. While the ventrolateral PAG induces long-lasting opioid analgesia connected also to immobility [62, 63]. Moreover, it seems that within PAG there is a least a crude somatotopy for analgesia in different parts of the body [64].

Several higher structures seem to be involved in the control of this system, among which prefrontal cortex, the ACC, the amygdala, the cingulate gyrus, the insular cortices and the hypothalamus [16, 65].

Even though it has been extensively studied and proved to be potent, the pain inhibition obtained by activating the PAG/DRN system has been challenging to utilize due to the involvement of this area in several other physiological functions than pain. Subsequently, it is still an open question whether this endogenous analgesic system can be efficiently and systematically used for pain therapies or not.

Attempts to treat persistent pain

Current pain therapies

With *physiological pain* or nociceptive pain, we refer to nociceptive acute pain and inflammatory pain which are adaptive and protective (both somatic and visceral). In contrast, *pathological pain* refers to maladaptive and non-productive mechanisms which are usually the result of both central and peripheral pathologies or plastic changes of the nervous system [66]. *Acute pain* is characterised by a relatively quick relief in contrast to *chronic pain* which refers to a continuous or intermittent pain condition which persists for a long duration. Chronic pain may be both physiological, if induced by an adaptive inflammatory reaction, or pathological, if persistent even after the trigger is no longer present due to internal complex modifications of the pain system. Several chronic pain conditions are also often accompanied by *hyperalgesia*, which is an increased pain sensation in response to a nociceptive stimulus [67].

Currently, available pain treatments are very diversified, and they might also be used in combination with other therapies. Their efficacy varies depending on the clinical condition, the gender and at the individual level.

Drug therapy. It is the most widespread therapy and the first approach to treating pain. However, drugs are less efficient for persistent pain disorders. The most powerful analgesics currently available are opioids, which act on opioid receptors

in the brain. Even though their effect is considered very strong, major issues relate to the use of these substances such as sedation and the development of addiction or tolerance, and other serious side effects [68-70]. In addition, their effect is highly variable and unpredictable, even causing hyperalgesia (an increased pain sensation in response to a nociceptive stimulus) in some individuals [71]. Alternative drugs include nonsteroidal anti-inflammatory drugs, which reduce inflammation by inhibiting the synthesis of specific molecules, and a very broad group called "adjuvant analgesics" which are usually used for treatments of other diseases, such as depression [72-74].

Trigger points injections. It is usually used to treat muscle pain, fibromyalgia, or pain due to muscle tension if the patient does not respond positively to other less invasive treatments. It involves the injection of local anaesthetics into the area which is recognized as the trigger point for the painful condition. It can provide potent pain relief but is limited to specific kinds of pain conditions [75].

Electrical/magnetic brain stimulations. It includes the interference of artificial electrical or magnetic signals with endogenous individual neural signalling. They include both invasive implants in the central nervous system (both in the brain and the spinal cord) and peripheral nervous system as well as non-invasive techniques such as transcutaneous electrical nerve stimulation therapy (TENS) or repetitive transcranial magnetic stimulation (rTMS). The effects are very different depending on the region involved in the therapy and often provoke side effects due to the accompanying modulation of alternative physiological pathways [76].

Intrathecal Drug Delivery. It involves the implantation of a pump below the skin through which it is possible to control the release of drugs directly into the intrathecal space around the spinal cord to block the pain signal. Since it is an invasive technique, it is possible to obtain a strong analgesic effect using a much smaller amount of the drug in comparison to an oral administration and therefore reduced side effects [77]. However, there are complications with the use of this technique such as overdose, withdrawal, cerebrospinal fluid leak or flow blockage [78].

Other therapies. Physical therapies and exercise, such as stretching, can reduce inflammatory symptoms and alleviate pain, but also induce the release of endorphins which is an endogenous painkiller [79]. Psychological therapies or mind-body control therapies can be useful ways to control pain for several patients [80]. They mainly aim to reduce stress which might be the cause of certain pain conditions.

Despite the huge variety of treatments for pain, often these therapies lack reliable efficacy, or they cause the development of tolerance or severe side effects. So, there is still a huge need for better analgesic therapies, in particular for intolerable persistent pain.

Current research for pain treatments

Many pain research studies try to find alternative therapies or approaches to solve the need for better analgesics.

Alternative drug targets. Improvements in our knowledge of nociceptive transmission and in particular the molecules and mechanisms involved in the development of inflammatory and neuropathic pain conditions open the possibility to find novel drug targets. Examples are nerve growth factor [81], sodium, chloride [82], hyperpolarization-activated cyclic nucleotide-gated ion channels [83], or calcium-sensor proteins [84]

Improvements of the existing therapies. Synthetic design of modified drugs (such as opioids, [85]) or electrical/magnetic modulation devices [86-88] which act more specifically in certain neural pathways might increase the analgesic/side effect ratio.

Localized analgesic delivery. To reduce side effects and optimize the analgesic effects, one obvious solution would be to move toward localised therapies directed toward the area involved in pain generation. Numerous drug delivery vehicles, such as liposomes or nanoparticles, have been proposed and tested to induce a sustained local analgesic drug release [89, 90]. However, the precise administration of drug delivery vehicles may still pose a significant problem.

Stem cell therapy. This therapy is based on the idea of transplanting stem cells into a specific brain region to restore pre-pathological conditions. An example was reported by Bráz et al. in which it was shown that GABAergic neuron precursors implanted in mouse spinal cord could improve neuropathic pain conditions [91].

Precision medicine. This includes a personalized pain treatment taking into consideration several aspects such as clinical, diagnostic, genomics, proteomics, or lifestyle information of a single patient. This approach is considered to have a huge potential for improved efficacy of pain therapies. However, to be successful there is a need for biomarkers for specific pain conditions. In fact, pain conditions and diagnoses are based mainly on self-reported symptoms, unlike other diseases [92]. The hunt for biomarkers for pain conditions has been very extensive, but still, there is no consensus about specific biomarkers, which relate to pain symptoms intensity or can predict the risk for the development of chronic pain conditions [93]. Because of the complex nature of pain, one possibility would be the use of a variety of biomarkers that together would construct a patient profile for different pain conditions.

Limitations in animal models of pain

In recent years, there has been overall modest progress in the pain field despite extensive efforts. Several analgesic treatments which were having promising results in basic research failed in clinical settings [94, 95]. Due to the poor success of this

translation, pain models have been questioned from different angles [95-97]. For instance, *in vitro* systems such as tissue slices or anaesthetized animals are distant from the normal physiological state [98]. Therefore, they do not represent the complexity of the dynamic spatial-temporal neuronal activity patterns in thalamic and cortical structures underlying pain perception and could lead to incomplete or even erroneous information. In this perspective, the use of awake freely moving animal models could recapitulate better the physiological mechanisms underlying pain and analgesia [99, 100]. However, even when awake animal models are used, the assessed variables need to represent as closely as possible the multiple dimensions of a clinical pain condition, including the mechanisms, time course, or cognitive changes. For example, it has been shown that hyperalgesia induced with ultraviolet B (UVB) light is a translational model since it has similar features in humans and animals [101].

Another aspect of fundamental importance is the subjectivity of pain. Since the same nociceptive stimuli can result in different painful experiences (due to different internal processing and the specific context), it is a big challenge to measure and compare results, especially when animal models are used. There is therefore the need for a direct and more reliable measure of pain magnitude in animals.

Notably, classical pain-assessment animal models have often been based on indirect measures of pain such as behavioural tests or motor measurements (e.g., withdrawal reflexes) which do not assess pain perception [102-104]. These motor-related assessments can be misleading since numerous examples show that they depend on neuronal mechanisms different to those that contribute to the perception of pain [27, 105, 106].

Several research groups recently employed changes in the electrical oscillatory brain activity as a pain biomarker, evaluating the variation within the different frequency bands during different pain conditions [107-111]. Nevertheless, the results have often been contradictory, and the use of these methods is debated [112-114].

An alternative way to obtain a direct measure of pain felt by an animal can be done by recording nociceptive-evoked signals in different brain areas [27]. In particular, the signals recorded in S1 retain information about pain location and intensity [24, 25]. In addition, it has been shown that nociceptive-evoked local field potentials recorded in S1 of rats and humans have similar features [26, 27]. Therefore, the S1 seems an ideal area in the brain to obtain a direct measure of pain-related nociceptive signals and to increase the chances for a successful translation of research findings from animal to human.

Deep brain stimulation

Brief historical overview of electrical brain stimulation

The first written evidence of the use of bioelectricity for electrotherapy dates back to ancient Greece where the torpedo fish was used to treat arthritis, headaches, pain and local swelling [115]. The use of this technique was described to induce a sensation of numbness accompanied by temporary pain inhibition and probably was known even from earlier civilizations such as the Egyptians [116].

In the 19th century, after brain electrical stimulation pioneering studies conducted by Luigi Rolando [117] and Pierre Fluorens [118], Luigi Galvani first discovered that muscles and nerves were excitable from electrical sparks. Subsequently, several studies, triggered also by the improvements in brain stimulation technologies, were done both in humans and animals. Of relevance are the studies of Wilder Penfield and Edwin Boldrey, previously mentioned, which led to the description of the somatosensory and motor homunculus. Walter Rudolf Hess [119], Robert Galbraith Heath [120], and José Delgado [121] demonstrated with the use of chronic electrode implants composed of straight wires that it was possible to elicit emotional responses when deeply implanted in the brain.

One of the modern therapeutic applications of electrical brain stimulation is a technique named “deep brain stimulation” (DBS) which involves the surgical chronic implantation of electrodes in the deep tissue of the brain. The delivery of electrical pulses in specific areas and with specific stimulation targets is used to treat or ameliorate the symptoms of a variety of neurological diseases such as Parkinson's disease, dystonia, obsessive-compulsive disorder, epilepsy, major depression, or chronic pain [122].

DBS probes development and state-of-the-art

Deep brain stimulation was initially utilized to localize the brain areas to be removed with ablative surgery starting in the 30s.

The first important technological advance which revolutionized the neurosurgical field was the advent of stereotactic surgery in 1947 [123] and the concomitant development of brain imaging techniques which allowed the 3D location of different brain areas.

Early attempts of chronic stimulation of deep targets were used for chronic pain and neuropsychiatric disorders in the late 40s and 50s [124, 125]. However, the patients had electrodes inserted in the brain while the wires were simply protruding from the skull. Subsequently, implantable stimulators for pacemakers were developed by Medtronic in the early 60s. Initially, these stimulators were utilized by

neurosurgeons mainly for pain inhibition, but then expanded to other brain diseases in the 70s. These first pacemakers had considerable technological limitations, but they gradually improved. A breakthrough was the advent of the fully internalised DBS with long-life lithium batteries during the 80s.

The first FDA approval for DBS use was granted in 1997 to Medtronic for tremors related to Parkinson´s disease and essential tremors. Later, other companies started to enter the same business and tried to optimise several aspects of the stimulation technology to make it more efficient and safer [126].

When looking at the technological advances and current state-of-the-art, there are several aspects to be taken into consideration.

Size and material. The probe for deep stimulation needs to be biocompatible, durable, and able to conduct an appropriate amount of current. Platinum-iridium and gold are the most commonly used conductive materials because of their minimal toxicity and low chemical reactivity with the tissue. The diameter of the commercially available probes for brain stimulation is in the mm scale and they are much harder than the soft brain tissue. Even though they are generally considered safe, they provoke significant local damage and tissue reactions due to their big size and stiffness [127].

Design. The classical electrodes for DBS stimulation are composed of 4 ring-shaped contacts in mm scale along a cylindrical rod. This design results in a too-low specificity of stimulation to address most brain areas. There are several companies and laboratories which are developing new designs with smaller and higher numbers of stimulation contacts along the electrode shank [128, 129]. Nonetheless, this development raised new programming challenges to be solved. In addition, these designs still caused relatively large stimulation fields with restricted opportunities for spatial tuning.

Types of stimulation. It has been observed that different waveform shapes (anodic/cathodic or symmetrical/asymmetrical) and stimulation parameters (frequency, current, pulse width, burst of stimulation) can elicit different physiological results. The optimal stimulation paradigm can vary depending on each specific condition. Also, while early DBS used just monopolar stimulation, lately several other stimulation patterns are under investigation, such as bipolar, alternating, directional or multiple-level stimulations [126, 130]. Directional stimulation, for instance, has been shown to reduce the side effects by localizing the current spread to a specific part of the brain reducing the activation of unwanted physiological networks [129, 130].

Closed-loop technology. The possibility to regulate the stimulation pattern based on a feedback signal is currently considered to have a great potential to minimize the side effect and adjust the stimulation in a patient-specific way over time. The challenge is to find reliable biomarkers for different brain diseases which can ideally

predict the surge of symptoms and activate the stimulation before these appear. For motor diseases, the use of accelerometers has been shown to be an efficient system to regulate DBS [131, 132]. For other diseases changes in the frequency bands of local field potential signals recorded from the implanted electrodes have been identified, but more basic research is needed to fully exploit the potential of a closed-loop technology [133].

Neuroimaging. One of the major keys to a successful DBS treatment is that the probe needs to be implanted in the exact brain area to be able to activate the specific neural network of interest while avoiding concomitant side effects. In the last decades, there have been a lot of improvements which allowed a higher precision in the presurgical target localization and the postsurgical probe localization. This is important for planning the implantation of DBS probes, but also to acquire data about the anatomical structure and the effect of the stimulation to further improve the treatments [134].

Deep Brain Stimulation in the midbrain for pain inhibition

The implantation of microelectrodes for DBS in the midbrain has been demonstrated to successfully alleviate pain in rats [135] and it was subsequently confirmed in other species and humans starting in the 70s [51, 136]. The discovery that endogenous midbrain centres in periaqueductal grey and dorsal raphe nuclei can induce a strong inhibition of pain perception raised the enthusiasm and curiosity of the scientific community. Several research groups conducted various studies to reveal the mechanisms of action of the observed phenomena and to fully exploit the potential of this area [39, 51, 137-139].

However, after many years of studies, the clinical outcome of this therapy has never reached reliable and efficient levels. Even though many aspects of the basic mechanisms of these structures have been clarified, the clinical use of PAG/DRN as a DBS target has been decreasing in the last decades favouring other types of pain therapies [140, 141]. One plausible explanation might be the use of inappropriately sized stimulation probes for this extremely complex neural structure involved in numerous physiological functions. In most cases, these probes included four separate channels resulting in a limited specificity of stimulation [129]. Even though it has been suggested that rat PAG/DRN include some “pure” analgesic spots [52, 142] and there are several cases in humans of successful implantations, a precise mapping of PAG is still missing. Therefore, a small misplacement or migration of the electrodes outside of these analgesic areas might have resulted in the activation of networks involved in other behavioural responses [143-146], leading to several unsuccessful results and the unreliability of this pain therapy.

Moreover, both in the clinical and experimental settings, the analgesic effect often decayed [147]. The reasons might correlate to possible damage to the neural tissue

surrounding the probe (due to the stiffness and big size of the electrodes used) [127, 148], fatigue of the system, or plastic changes which lead to adaptation/tolerance (or a combination of these).

Tissue reactions to brain implants

Electrical-neural interfaces are devices connecting the brain to the external world by recording and/or stimulating the neural tissue. They hold great potential for both revealing brain physiological mechanisms and for treating pathologies.

Currently, a variety of technologies exist to interface the brain to computers, both non-invasive (not in direct contact with the neural tissue) and invasive (directly in contact with the neural tissue). Even if a non-invasive probe would be ideal, they currently do not provide the spatial resolution that an invasive probe can achieve, which is needed in several cases [149].

Invasive probe technology has improved during the latest decades (especially in the number of electrode contacts/probes) [150]. However, most of the developed probes still share a common unsolved problem: the brain tissue reaction to foreign bodies. These responses can compromise the probe function as well as the physiological state of the recorded/stimulated nearby neural tissue.

Mechanisms and time-course

Brain inflammatory processes can be activated both by chemical and mechanical stressors leading to neuronal death, and the formation of an encapsulation sheet which reduces the performance of an electrode [151].

While the tissue reactions caused by the probe material have been mostly overcome using inert and durable materials (such as gold, platinum, and platinum-iridium), there are two different events causing inflammatory responses which are much more difficult to control and resolve.

1. Insertion of the probe in the brain tissue. To reach a specific area, a neural probe needs to penetrate the brain tissue. This insertion is often the cause of acute trauma including cellular and blood vessel disruption, and tissue compression [152-155].

2. Micromotions between the implant and the brain. Stiff implants tethered to the skull follow the skull movements, while the soft brain tissue, which floats in the cerebrospinal fluid, is slower in following these movements. This mismatch is assumed to cause chronic trauma and to sustain an inflammatory response which leads to the formation of a cellular sheet around the external body also referred to as a “glial scar” [156-159].

Astrocytes and microglia are assumed to be the main cell types involved in the tissue reaction in the central nervous system [156], although other types of cells may also play a role [160]. Astrocytes are star-shaped cells with several functions among which the contribution to the blood-brain-barrier, sustaining neurons, homeostasis maintenance, and neural tissue repairing [161]. Microglia are resident macrophages in the brain which, in physiological conditions, show a branched shape with arms constantly surveying the surrounding tissue [161].

When an external body, such as a neural probe, is introduced into the brain it causes cellular damage. The injury is detected by the local microglia in the nearby tissue which sends their branches toward the damaged area within 30 minutes trying to encapsulate the foreign body [162]. The microglia also release cytokines to recruit more microglia and monocyte-derived macrophages from the bloodstream [163]. Within 12 hours after the injury, macrophages, and proliferating microglia (which change their morphology to a more compact shape, and which became phagocytic to clear the tissue from debris and toxic substances) can be seen at the interface of the implanted body (figure 2) [164, 165]. Astrocytes have a slower reaction and they appear to be in an activated state around 12 hours from the damage, peaking about 1 week after [166].

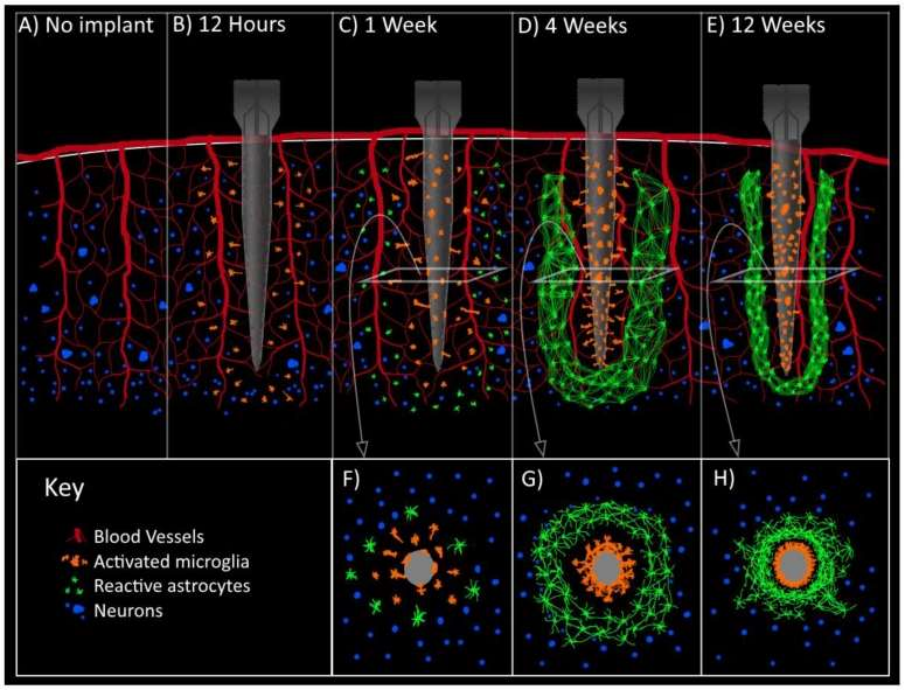

Figure 2. Timecourse and mechanism of neural tissue reactions to a brain implant.
Schematic an inflammatory response progression and the main cell type response to a neural interface at different time points. The schematic shows the cortical surface both in a sagittal (**a-e**) or coronal (**f-h**) perspective. Reproduced with permission from Creative Commons´ open access policy from [167].

During acute injuries, the inflammatory response decline at this point. However, the continued presence of the electrode within the tissue usually leads to a chronic inflammatory tissue response and to the formation of a dense astrocytic layer surrounding the phagocytic cells around the implant about 2-3 weeks after the foreign body insertion [153, 164, 167]. This layer called "glial scar" may have the function to protect the blood-brain barrier and preventing lymphocyte infiltration [168]. In addition, during the inflammatory process, the microglia produce molecules which are known to have a neurotoxic effect [169]. This may be a contributing factor to the neural death observed around the implants [158, 170].

Even though inflammation may have beneficial effects to a certain extent [171], the formation of an encapsulation sheet and neuronal death inevitably cause reduced functionality or even a failure of an implanted probe. Methods to reduce tissue reactions to brain implants might therefore improve the efficiency of the neural interface.

Strategies to minimize foreign body responses in the brain

Different strategies have been proposed to minimise the acute and chronic inflammatory response to an external body.

Reduced electrode size and use of biocompatible materials. Probes with a reduced size have been shown to be more biocompatible [172, 173]. In addition, the use of inert and durable materials for the electrode composition and insulating layer is also essential to avoid inflammation response triggered by released chemical molecules or electrode debris.

Refined probe insertion methods. The insertion speed of a probe can influence tissue damage. In fact, a slow insertion speed seems to cause less tissue damage allowing the tissue to rearrange around the inserted body [174]. However, while penetrating the meninges a slow penetration can cause dimpling and tissue compression [153]. So, a compromise in which a fast speed for penetrating the pia mater and a slower speed while approaching the target area may be better. Other techniques aiming to reduce the friction and drag forces between the probe and the tissue might be used to reduce tissue damage, such as the use of a fine tip to avoid tissue compression [155, 175] or an ice coating on the probe to reduce friction forces [176].

Use of flexible probes. As previously mentioned, to avoid chronic inflammation due to the relative movements between the brain and the neural probe, it is preferable to have a flexible probe [172, 177, 178] with a similar density of the brain tissue [179] and that can "float" and follow its movement [159, 180, 181]. However, these probes need structural support during implantation. Some examples to overcome this problem are: i) the use of guiding rods removed after implantation [182, 183]; ii) materials which can change their flexibility in different conditions (in response to wet environments, temperature variation, or chemically controlled triggers) [184-

186]; iii) bioresorbable coatings which are stiff when dry but once implanted dissolve in the brain leaving the flexible probe [187-189]. Among the bioresorbable material, gelatine has been shown to also reduce insertion trauma and promote the healing of the blood barrier [154].

Use of anti-inflammatory drugs. The use of anti-inflammatory and neuroprotective drugs has been used to decrease local inflammation and avoid neural death [190, 191]. One of the used drugs in experimental settings is minocycline, a second-generation tetracycline antibiotic which has been shown to inhibit the activation and proliferation of the microglia [192, 193]. Several delivery methods have also been tested among which are the use of microfluidic channels [194, 195] or the incorporation of the molecules on biodegradable coatings added on the probe [188, 196]. The introduction of a cannula allows a sustained release of the drug but increases the probe dimension (and therefore the risk of increased damage during and after implantation). On the contrary, biodegradable coatings release most of the drug content within the dissolution time of the coating material, which can vary vastly from material to material. Other methods have been suggested such as electrical stimulation-controlled release from a conducting polymer or the use of drug-loaded Poly(Lactic-co-Glycolic Acid) or PLGA-nanoparticles which sustains the drug release over weeks [90].

Even if some of these approaches have limitations or introduce new problems to solve, they have been proven to reduce acute inflammation. Further improvements or the use of a combination of these strategies may thus be the best solution to minimise the tissue reaction.

Aims

The purpose of this thesis was to evaluate whether the PAG/DRN anti-nociceptive control can be reliably and consistently exploited to treat persistent pain employing a new technique for high-definition brain stimulation (HDBS).

The specific aims of this thesis were:

Aim I

To assess whether HDBS in PAG/DRN can be used to specifically inhibit the transmission of nociceptive information to S1 (receiving sensory-discriminative nociceptive information) without provoking adverse side effects in normal conditions and during hyperalgesia (papers I and II).

Aim II

To clarify if HDBS in PAG/DRN is equally effective on sensory-discriminative, affective, and motor aspects of pain in normal conditions and during hyperalgesia (paper III).

Aim III

To evaluate the reliability, selectivity, and sustainability of HDBS in PAG/DRN and biocompatibility of implanted microelectrode cluster (papers I-III).

Aim IV

To develop a method for reducing glial reactions to brain implants (paper IV).

Methods

In this section, there will be a brief description of the techniques developed and used in the papers. For more details, I kindly refer the reader to the respective papers.

Neural interfaces

All the probes employed in different experiments were developed in our laboratory. A summary of the implants used in different papers can be seen in figure 3.

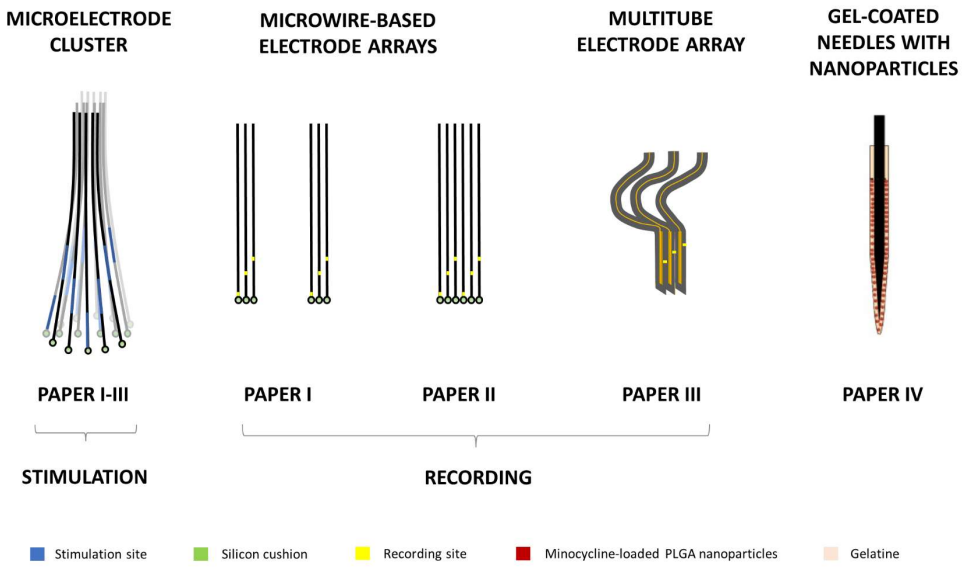

Figure 3. Schematic summary of the implants used in different papers.
The schematic is not to scale. The stimulation and recording probes were embedded in a gelatine vehicle for structural support before implantation, but the gelatine layers are not shown here.

Stimulation and recording probes were based on ultra-flexible microwires embedded in gelatine vehicles. The choice to use ultrathin and highly flexible microwires is related to their higher biocompatibility, which enables long-term

recordings [157, 172, 177, 178, 183]. These microwires need structural support to be implanted, which in this thesis was achieved by coating them with gelatine. Gelatine is a protein which derives from the breakdown of natural collagen and, when dried, allows the mechanical support for implanting the microwires. Once in the brain tissue, it swells and then dissolves and can be easily metabolised. In addition, it has been shown that gelatine reduces the ubiquitous loss of neurons nearby implanted probes [157], reduces microglial activation [157] and improves the blood-brain barrier restoration after injuries [154].

The multitube electrode array is a novel technique for brain recordings where the microelectrodes become flexible after implantation and assume a density close to that of the brain tissue [197]. This feature makes this array highly biocompatible and appropriate for long-term recordings. Its design was modified in this thesis and adapted for recording in different layers of the S1 and ACC.

In a parallel study, we also evaluated the effects on the glial reaction of adding minocycline-loaded PLGA nanoparticles to implants coated with gelatine to investigate possibilities to further reduce the inflammatory response at the site of implantation.

Stimulation microelectrode cluster

Design. The probe for HDBS was based on a cluster of 16 ultra-flexible microwires implanted in the target area, wherein the deinsulated contacts were located at different depths. This design enables granular microstimulation in a 3D volume.

Manufacturing. Platinum iridium wires of 12.7 µm diameter were insulated through chemical vapour deposition with a layer of 4 µm of Parylene C, then deinsulated for 300 µm at three alternative distances from the distal end of the electrode (0, 300, 600 µm) in a laser milling system (LaserMill 50, standard micromilling system, New Wave Research Inc., USA). Silicon cushions with ~40 µm diameters were introduced at the distal tip of each microelectrode to further protect the tissue and reduce local bleedings. Five or six wires were then arranged on a flat anti-adherent surface and freeze-fixated by spraying a 10% gelatine solution. The wires were then stacked and inserted in a cylindrical plexiglass mould (size of the inner compartment: 9 mm long and 350 µm diameter) and narrowing down distally. 30% gelatine solution heated at 50°C was thereafter injected into the mould to obtain a gelatine needle-like probe with wires embedded in it. The proximal part of the wires, which was protruding from the gelatine vehicle, was deinsulated with a butane flame and soldered to an omnetic connector together with a 25 µm platinum wire for grounding. All the deinsulated parts of the wires were covered with medical grade epoxy (Epoxy Technology, EPO-TEK OG198-54 and 55, USA) and finally, the probe was released from the mould (figure 4). Before implantation, the single wire impedance was measured with a NanoZ impedance tester (White Matter LCC,

nanoZ v1.4.0 software, USA) by inserting the probes in a saline solution at 4°C. Probes with more than 2 non-functional channels were not used further.

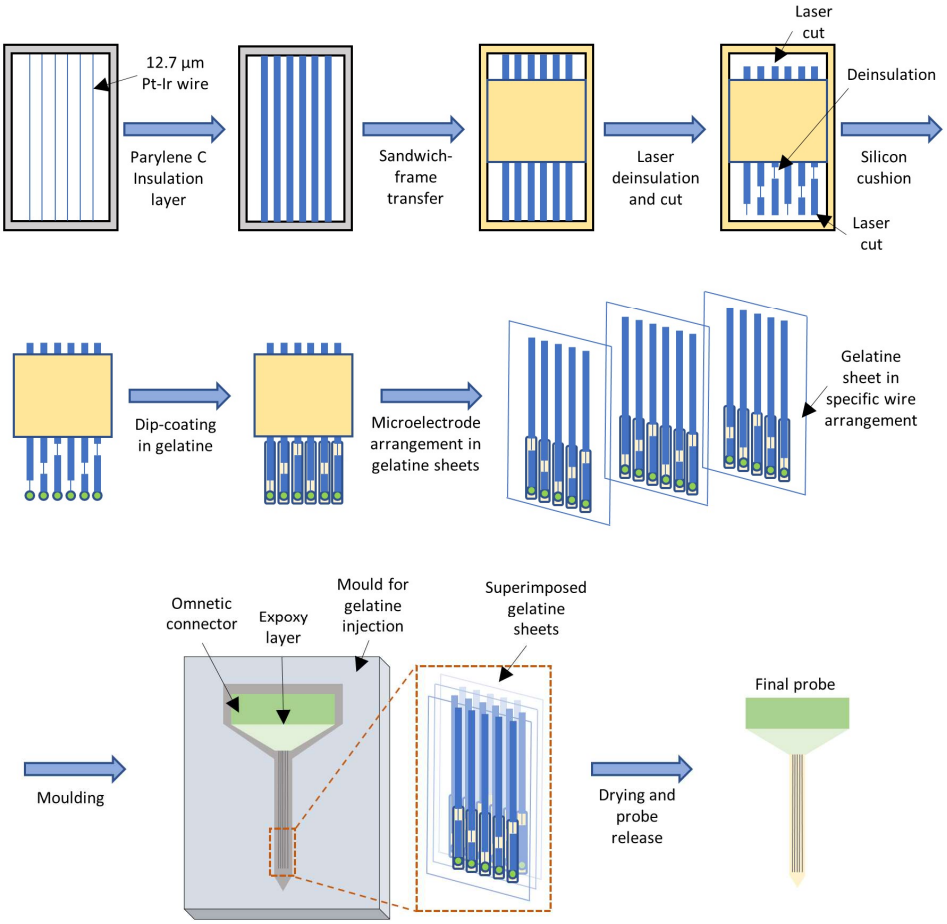

Figure 4. Schematic of the stimulation probe manufacture steps (adapted from paper I).

Recording electrodes

Microwire-based electrode array

Design. Microwire-based electrodes were used for local field potential (FP) and multiunit activity (MUA) long-term recording in papers I and II. The aim of paper I was to investigate the general PAG/DRN stimulation effect on all four limbs of the animal. Therefore, microelectrodes for recordings were implanted in both the forepaw and hind limb primary somatosensory cortices. In paper II, the microelectrodes for recordings were implanted in the hind limb area of S1.

Manufacturing. As for microelectrodes for stimulation, platinum-iridium wires of 12.7 µm diameter were insulated with a layer of 4 µm Parylene C through vapour deposition. The wires were deinsulated for 50 µm at different alternative distances from the tip and cut at the proximal and distal parts via a laser milling system. A silicon cushion of about 40 µm was manually added at the distal end of each wire. They were then placed on an anti-adherent surface and fixated with a drop of 30% gelatine solution. In paper I, two different bundles composed of three microelectrodes (with different axial locations) were manufactured for the hind limb and front limb area, respectively. In paper II, a single bundle composed of six microelectrodes (with different axial locations) was manufactured for the hind limb area. Their distal ends were then dipped in the same gelatine solution to let the wires adhere to each other and coat them with a gelatine layer. After drying at room temperature, the proximal ends of the wires were de-insulated with a butane flame and soldered to an omnetic contact together with a ground wire and the deinsulated parts of the wires were covered with epoxy.

Multitube electrode array

Design. To improve the quality of neural recordings, a novel probe was developed by the modification of a single tube electrode developed in our laboratory [197] and used in paper III. This electrode consists of a gold microwire coated with glucose and insulated with Parylene and an orifice along the shank. The glucose was hard enough to allow brain penetration, but once inside the neural tissue it dissolves leaving a very flexible and low-density construction for neuronal recordings where the gold microwire is in contact with the neuron activity through the orifice and the extracellular fluid. Three tube electrodes were held together by gelatine during implantation (figure 5).

Manufacturing. Three 12.7 µm gold wires were soldered to an omnetic contact and displaced onto a frame parallel to each other. The omnetic contacts and the proximalmost 4 mm of the wires were masked while the rest of the wires were covered by a layer of glucose by electrospraying to reach a diameter of 22-27 µm. After the mask removal, the electrodes were insulated with a layer of 2 µm of Parylene C through vapour deposition. The wires were then obliquely cut at the distal end 3 mm from the proximal end of the glucose layer with a scalpel. The proximal glucose-free parts of the wires were bent to create a flexible zone. The wires were then insulated with an additional outer layer Parylene C (2 µm). Thereafter an orifice in the insulation material of approximately 35 µm in diameter at 3 alternative distances was created by evaporation in a laser milling system. Subsequently, the glucose-coated parts of the wires were aligned and attached to each other by adding a layer of 10% gelatine solution, while the bent glucose-free parts were left separate (figure 5). At this stage, a 100 µm stainless steel needle was glued through a small drop of 10% gelatine solution at the border of the glucose-free and glucose-coated part of the probe and secured in the same way onto the

omnetic contact to ensure mechanical strength during the surgical insertion in the brain. Finally, a 50 µm ground silver wire was soldered to the omnetic contact.

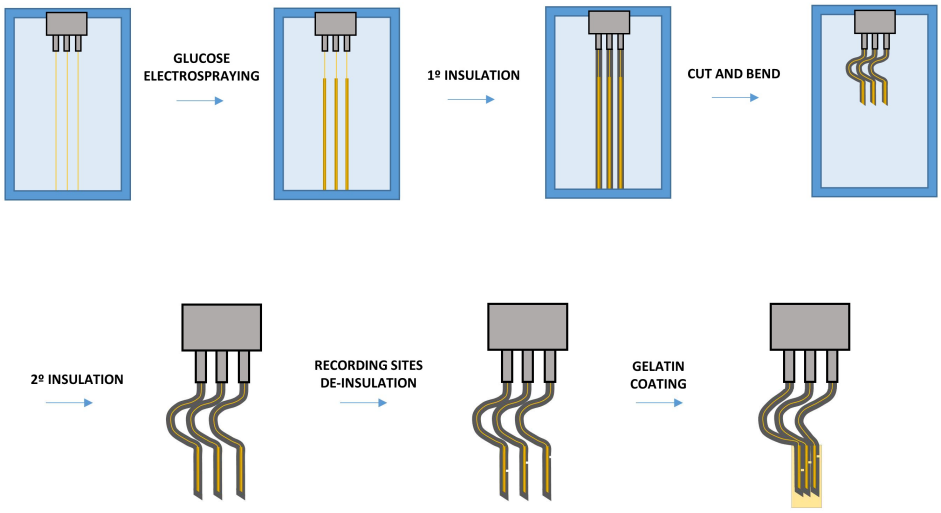

Figure 5. Schematic multitube recording array manufacture steps.

Electrocorticography (ECoG)

In paper II, ECoG electrodes composed of 25 µm platinum insulated with a 4 µm thick Parylene C coating and deinsulated for 500 µm at the distal end were also soldered to the same omnetic contact as the microwire-based recording array.

Summary

A summary of the different recording electrodes used in the different papers to answer different research questions is described in table 1.

Table 1. Properties of microelectrodes used for recordings in the different studies.

	Paper I	**Paper II**	**Paper III**
Type of implant	2 microwire-based arrays comprising 3 microwires each	1 microwire-based array comprising 6 microwires 1 ECoG	2 multitube arrays comprising 3 tubes each
Conductive material	Platinum-Iridium	Platinum-Iridium Platinum	Gold
Implantation area	S1 – hind limb S1 – front limb	S1 – hind limb Frontal cortex	S1 – hind limb ACC
Depth from cortex (µm)	750, 1000 or 1400	750, 1000 or 1400 On top of dura mater	750, 1100, and 1600 1000, 1500, and 2000

Gelatine-coated needles with minocycline-loaded nanoparticles

Design. Even if the probes described above were expected to have minimal impact on neural tissue due to the ultra-flexible microwires and protective gelatine coatings, an additional strategy to minimize tissue responses was investigated in this thesis. This method consisted of the use of drug-loaded PLGA nanoparticles embedded in the gelatine surrounding the brain implants for local and sustained drug release. The choice of using drug-loaded PLGA nanoparticles is due to a previous study which showed that sustained drug release for several weeks can be achieved *in vitro* [90]. The chosen drug was minocycline, an antibiotic with anti-inflammatory and neuroprotective properties.

Manufacturing. The PLGA-nanoparticles loaded with minocycline were prepared through the emulsification-solvent diffusion technique as described in [90]. The obtained nanoparticles were ~220 nm in size and had a drug content of 1.12 ± 0.01%. Stiff and sharp stainless-steel needles (diameter of 100 μm) coated with minocycline-loaded nanoparticles embedded in gelatine were used to evaluate their effects on the inflammatory response. In brief, the needles were insulated with a layer of 4 μm Parylene C through vapour deposition. They were then dipped in a solution of 30% gelatine dissolved in artificial cerebrospinal fluid heated at 60 °C and kept in dark and dry conditions (less than 1% humidity) at room temperature. The gelatine-coated needles were then immersed in a suspension containing the nanoparticles at room temperature to allow the gelatine to swell (but not dissolve) and incorporate the nanoparticles by diffusion. They were then left to dry and stored in the same conditions (figure 6). Gelatine-coated needles without nanoparticles were also manufactured as controls.

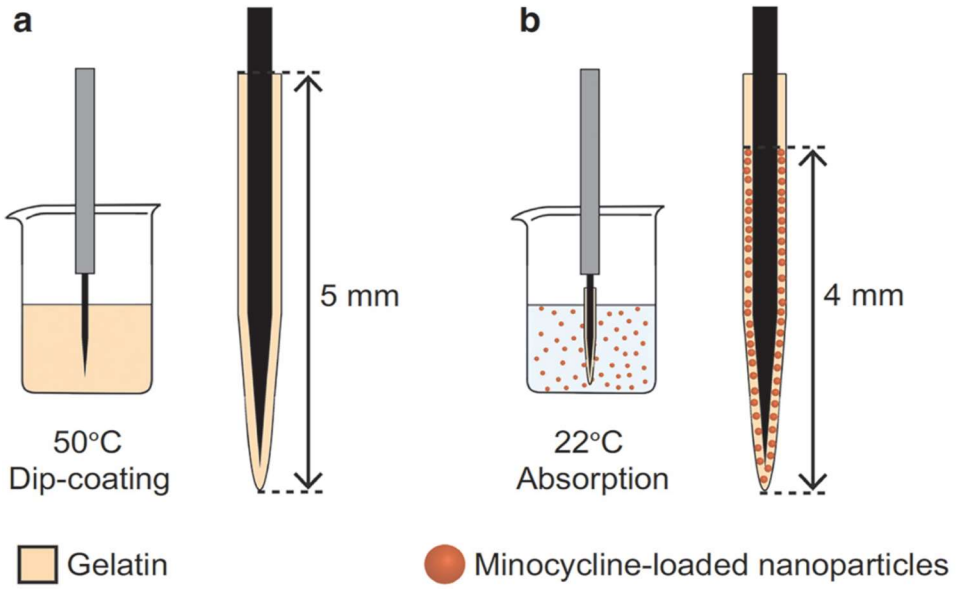

Figure 6. Schematic method to obtain drug-loaded nanoparticles in gelatin-coated needles.
(a) Dip-coating of the needle in a gelatin solution to obtain a uniform gelatin layer of 5 mm around the needle. (b) Dip-coating of the gelatin-coated needle in an aqueous suspension containing the minocycline-loaded nanoparticles resulting in the absorption of the nanoparticles in the gelatine coating.

The gelatine-coated needle diameter was uniform, with a coating thickness of 4.8 ± 0.9 µm (only gelatine) and 9.1 ± 1.2 µm (gelatine with incorporated nanoparticles). It was therefore possible to calculate the absorbed volume (~85 nl) and a drug content of ~1 µg nanoparticles containing ~34 ng minocycline.

Animals and surgical implantations

All the procedures were approved by the Malmö/Lund Animal Ethics Committee on Animal Experiments (ethical permits: M4480-18 for rat experiments; M61-13 for mouse experiments). All the animals were kept at constant temperature and humidity (21 °C and 65% humidity) in a 12-hour light/dark cycle and had constant access to food and water (with exception of the Catwalk experiments, in paper I, in which the rats were food deprived 16-20 h for a 5-7 days training period).

During the surgical procedures, the animals were anaesthetised using 2% isoflurane mixed with 40% O_2 and 60% N_2O and the isoflurane level was kept between 1-2% for the whole operation while the animal body was on a heated surface at a constant temperature of 37 °C.

Rat stimulation and recording probe implantations (papers I-III)

The surgical procedures for these implantations were similar for all studies, but the implanted probes (figure 3) and the coordinates (table 2) varied.

Table 2. Coordinates used for recording and stimulation probe implantations in rats.
Coordinates are indicated in mm with respect to Bregma. AP, anteroposterior; ML, mediolateral; DV, dorsoventral. *For all the recording electrodes, the DV refers to the location of the deepest deinsulated area of the probes from the cortical surface.

Paper I					
Probe	**Area**	**AP**	**ML**	**DV**	**Insertion Angle**
Stimulation	PAG/DRN	-8	0.0	-6.2	-30
Recording microwire array	S1 (hind limb)	-0.84	-2.4	-1.45*	0
Recording microwire array	S1 (front limb)	-0.84	-4.0	-1.45*	0
Paper II					
Stimulation	PAG/DRN	-7.8	0.0	-6.2	-30
Recording microwire array	S1 (hind limb)	-1.5	-2.4	-1.45*	0
Paper III					
Stimulation	PAG/DRN	-7.8	0.0	-6.2	-30
Recording multitube array	S1 (hind limb)	-1.5	-2.4	-1.6*	0
Recording multitube array	ACC	2.0	-0.8	-2*	0

After the head of female Sprague Dawley rats was shaved and fixated into a stereotactic frame, the skull was exposed, cleaned and calibration of the head position was performed with a stereotactic programmed micromanipulator (Neurostar, StereoDrive 4.0.0, Germany). Holes for 4 screws and ground wires (and ECoG electrodes for paper II), and craniotomies for the electrode insertion were performed with a drill. The dura was removed to reduce dimpling caused by the probe insertion in the brain. Then the probes were inserted with a programmed micromanipulator and secured to the skull with dental before the implantation of the following one. A two-speed insertion method was used to minimise tissue damage (more details can be found in table 3). ECoG and ground wires were placed on top of the dura mater in contact with the cerebrospinal fluid. Finally, the probes and the animal skull were covered by dental cement except for the omnetic contact left uncovered for electrical connections.

Table 3. Parameters used for probe insertions in the brain.

Probe	Pre-target (mm)	Speed to pre-target (µm/s)	Speed to target (µm/s)	Waiting time (minutes)
Stimulation probe	2	1000	100	3
Recording microwire array	0.45	1000	100	0
Recording multitube array	1	1000	100	0

Temgesic (0.01 mg/kg) and 5 ml of physiological saline were injected subcutaneously about 20 minutes before awakening for pain relief and hydration, respectively.

Mice coated-needles implantations (paper IV)

Male and female CX3CR-1^{GFP} transgenic mice (which express the green fluorescent protein, GFP, in the microglia) were shaved and their head was mounted on a stereotactic frame. After the skull was cleaned, ~1 mm diameter craniotomies were drilled (bilaterally, midways between Bregma and Lambda, and ~1 mm lateral to the midline). The gelatine-coated needles (controls and drug-loaded, one in each hemisphere) were cut at 3 mm and inserted in a glass capillary filled with paraffin oil to avoid water uptake and gelatine swelling before its penetration in the neural tissue. The capillary was mounted on a micromanipulator and the needles were implanted 3 mm below the cortical surface with a 500 µm/s insertion speed.

Assessment of effects induced by HDBS in PAG/DRN

The assessment of the analgesic effect of PAG/DRN stimulation on the nociceptive-evoked cortical responses and withdrawal rate as well as the assessment of other effects were performed through a series of experiments in which the variation of several parameters in different conditions was investigated, as listed below.

The set-up for electrophysiology and behavioural experimental procedures as well as the tactile/nociceptive stimulations were kept constant between different experimental sessions.

The rats were placed on a metal grid surface surrounded by a plexiglass cage (an exception was made for the animal movement tracking experiments in paper II, in which a black plate was used to enhance the contrast between the animals and the background) and habituated to the environment for at least 20 minutes before the beginning of the experimental sessions. The recording probes were connected to a multichannel neural data acquisition system and the stimulation probe to a current-

control stimulator regulated by an in-house developed MATLAB software package. The stimulations were delivered with biphasic charge-balanced squared pulses (2x50 μs) and at 50 Hz (except for a stimulation session in which the effect of different frequencies was investigated).

Nociceptive thermal stimulations were delivered by short pulses from a CO_2 laser in which the intensity of the pulse was regulated by its duration. Tactile stimulations were delivered through a magnetically triggered device. They were both connected to a stimulator providing a time stamp for the data acquisition system. The nociceptive threshold was defined as the lowest pulse eliciting $\geq$3/5 withdrawals and was measured before each experimental session for the paws of interest. A maximum pulse duration of 32 ms was used to avoid skin damage.

The laser and tactile stimulations were delivered to the paws contralateral to the location of the implanted S1 and ACC recording arrays (except for the cluster selection phase in paper I, where all the paws were analysed).

Stimulation microelectrode cluster selection (papers I-III)

After the implantation and spread of a surplus of microelectrodes in the PAG/DRN area, a subset of microelectrodes inducing reflex-analgesia without noticeable side effects was selected for each animal. The selected group of microelectrodes and stimulation current were kept the same thereafter in the following experimental sessions.

The methods used in this experimental phase slightly changed during the progression of the different studies to further improve the quality of the results or depending on the specific need of each study (see table 4).

Table 4. Differences during the stimulation microelectrode cluster selection among different papers.
PS, post-surgery.

	Week PS	Analysed area	Individual channel stimulation
Paper I	2-3	All the four paws	Same maximal current selected for all the channels
Paper II	2-3	Right hind paw	Individual maximal current selected for each channel
Paper III	2-5	Right hind paw	Individual maximal current selected for each channel

In paper I, each electrode was stimulated singularly using the same current level of 50 μA and its analgesic power was evaluated depending on the inhibition of withdrawal reflexes (elicited using laser stimulations at nociceptive thresholds) at this current. Elicited side effects were also noted. In papers II and III, this procedure was refined to further minimise the appearance of side effects during this explorative phase by selecting an individual maximal current for each channel based on the

stimulation-elicited behavioural responses. This maximal current was determined by increasing gradually (in steps of 10 μA) the stimulating current, from 10 to the appearance of side effects to a maximum of 50 μA. Thereafter, the single-channel reflex-analgesic ability was tested at the selected current. Based on the anti-nociceptive abilities of the single channels, various combinations of stimulation channels and currents were tested to obtain a powerful and side effect-free reflex-analgesia. The selected current, termed I_{max}, was the maximal current (always below 50 μA) that was possible to deliver with a specific microelectrode combination without inducing side effects.

Selection of stimulation parameters (paper I)

The choice to use a frequency of 50 Hz in the microelectrode selection phase was motivated by previous studies in the literature that most commonly used this frequency [52, 55, 198, 199]. However, reports of effects using different stimulation currents in the same animals were missing. Therefore, the analgesic effect of HDBS was investigated by recording nociceptive-evoked (n=16) cortical responses (FPs and MUA) in S1 (which relates to the perceived sensory-discriminative aspects of pain) and withdrawal reflexes with or without HDBS at different frequencies (5, 20, 50, 90, and 130 Hz) at I_{max}.

To evaluate if the HDBS effect was having a graded or all-or-nothing effect a similar experiment was done but with fixed stimulation frequency (50 Hz) and varying the stimulation intensity. The tested intensities were the individual intensities (30-50 μA) selected in the microelectrode cluster selection experimental phase (I_{max}), and 10 and 20 μA below I_{max}.

Both frequency and intensity experiments were done during weeks 4-5 post-surgery (PS) and repeated 3 times.

HDBS in comparison to morphine (paper I)

A comparison between morphine and HDBS in PAG/DRN was performed to benchmark the analgesic effect of this technique. Recordings of nociceptive-evoked cortical responses from S1 and withdrawal rate during 16 nociceptive stimulations (delivered onto the front and hind paws) were performed with or without PAG/DRN stimulation and during the administration of 1 or ~3 mg/kg subcutaneous morphine. The experiments were repeated 3 times and done during weeks 4-5 post-surgery (PS).

HDBS during hyperalgesia (papers I and III)

UVB-induction of hyperalgesia

As previously mentioned, UVB-induced hyperalgesia has been shown to be a translational model of pain since it shares a similar mechanism and time course with humans [101]. Therefore, it was chosen in this thesis as a model of persistent pain. In addition, this model gave the opportunity to analyse the primary hyperalgesia area (directly exposed to the UVB light) and the secondary hyperalgesia area (the area nearby the primary area).

After being anesthetised with isoflurane, the rats´ distal lateral part hind paw area (paper I) or the whole hind paw (paper III) contralateral to the S1 probe was exposed to an even field of UVB light. The energy delivered (regulated by the time of exposure and the proximity to the UVB lamp) was calculated to be at an intensity to induce strong hyperalgesia, but below the threshold which induces blisters (1.3 mJ/cm2). Laser Doppler flowmetry on the rats´ paws was done before the UVB light exposure and repeated after 2 days (in paper I) or after 1, 2, and 4 days (in paper III) to verify the induction of hyperalgesia.

HDBS effects on nociceptive-evoked cortical responses in hyperalgesia

HDBS in PAG/DRN analgesic efficacy was evaluated during hyperalgesia to investigate whether this technique was efficient during persistent changes in the pain system also. The experiments were performed during weeks 6-7 PS.

In paper I, the HDBS effect in primary hyperalgesia and secondary hyperalgesia were investigated. Nociceptive-evoked cortical responses (n=16, repeated 3 times per condition) were delivered during cortical recordings in S1 in control and during HDBS 2 days after UVB exposure. In paper III, the HDBS effect was evaluated both on S1 and ACC (which relates to the perceived affective aspects of pain, [31]) nociceptive-evoked responses (n=32 repeated 2 times per condition) in the primary hyperalgesia area-, 1 day after-, and 2 days after-UVB exposure and in control conditions.

HDBS during continuous stimulation (paper II)

Continuous stimulations of PAG/DRN were done to investigate the variability of the HDBS effect in PAG/DRN over prolonged stimulation time and adjustments of the stimulation current were introduced when a decay of effect was observed. A post-stimulation effect was also monitored.

Cortical S1 recordings and withdrawal rate assessments were done during 16 laser stimulations (delivered on the hind paws) with or without HDBS at different time points: 0, 15, 30, 60, 90, 120, 150, 180, 210, and 240 minutes from the beginning of the PAG/DRN stimulation and 0, 15 and 30 minutes after the end of the stimulation.

The same protocol was repeated in a different experimental session, but when 3 or more withdrawals were observed the current was increased in steps of 5 µA. These experimental sessions were performed during weeks 4-5 PS.

Long-term HDBS reliability (papers I-III)

An experimental session including 16 laser stimulations delivered onto the hind and front paws with or without HDBS in PAG/DRN during cortical recordings was repeated at 7-12 weeks PS prior to perfusion. HDBS was done using the same stimulation parameters as defined during the stimulation microelectrode cluster selection phase in the first weeks PS, to verify the analgesic stability in time. In paper I, 16 nociceptive stimulations were repeated 3 times for each condition.

Assessments of spontaneous cortical activity (paper I)

To verify the HDBS impact on the spontaneous activity and its comparison with morphine, one-two minute recordings of spontaneous MUA in S1 during inactive periods (e.g. when moving or grooming) were performed in control conditions, during HDBS PAG/DRN, and after subcutaneous injection of 1 mg/kg morphine between week 5-10 PS.

Assessments of motor behaviour (papers I and II)

Effects on gait in normal conditions and hyperalgesia (paper I)

The rats were trained to walk/run along a Catwalk system (Noldus Information Technology, The Netherlands) narrow track, the paw print intensities were analysed, and the speed of the animals was recorded with a video camera. After a training period (5-7 days), the rats repeated the run along the Catwalk 5 times in each condition (with or without HDBS) before and 2 days after UVB irradiation of the hind paw. The paw prints were automatically classified by the Catwalk program and visually validated and exported for further analysis.

Assessments of general activity in an open field (paper II)

The rats were left free to move in an enriched environment with food, water, nesting material or nothing separately displaced in different corners of a squared cage (figure 15a). Their behaviour was monitored with a recording camera placed above the cage with and without HDBS in PAG/DRN (1 hour per condition).

Assessments of ECoG (paper II)

Spontaneous ECoG recordings (with or without HDBS, 1 hour per condition) were performed to evaluate possible changes in the animals´ brain states which might be difficult to spot from a behavioural analysis. The recordings were performed in the same open field described in the previous section.

Specificity for nociception (paper I)

The effect of HDBS in PAG/DRN was evaluated also on tactile-evoked potentials to assess the specificity of the PAG/DRN stimulation for nociception.

Sixteen tactile stimulations were delivered onto the rat front and hind paws during cortical recordings in control conditions and during HDBS in PAG/DRN. The procedure was repeated three times. The experiment was performed in normal conditions (weeks 4-5 and 10-11 PS), and during hyperalgesia (weeks 6-7 PS).

Biocompatibility and probe placement

Immunofluorescence analysis (papers II-IV)

Standard perfusion and immunofluorescence staining protocols were used for histological analysis of the tissue reactions to different brain implants (stimulation probe in papers II and III; gelatine-coated needles in paper IV). Table 5 shows a summary of the primary antibodies used, while more details can be found in the single papers. To determine the stimulation probe placement (paper II), a reference point and the stimulation microelectrode tip were measured from tissue slices stained with GFAP and DAPI and inserted in a Waxholm brain atlas for visualization.

Table 5. Summary of primary antibodies used in papers II-IV.
CD68, Cluster of Differentiation 68; ED1, Ectodysplasin A; GFAP,Glial Fibrillary Acidic Protein; NeuN, neuronal nuclei; DAPI, 4′,6-diamidino-2-phenylindole.

Name	Target
CD68/ED1	Activated microglia and macrophages
GFAP	Activated astrocytes
NeuN	Neuronal nuclei
DAPI	Cell nuclei

Data analysis

After the data acquisition, all data processing was done using automated analysis based on custom-made Matlab or R scripts or using Nis-elements to avoid biases.

Evoked cortical responses (papers I-III)

Individual evoked FPs, sampled at 1kHz and low pass-filtered (<200 Hz), were preprocessed to remove artefacts, averaged and smoothened obtaining an estimate of the evoked FP per recording channel.

Evoked MUA, obtained from wideband recordings sampled at 40 kHz and high-passed filtered (>300 Hz), was estimated per channel as the smoothened normalised z score, obtained from spike times detected by applying a threshold of three minus the estimated noise level of the highpass filtered recordings.

In paper I, the channel with the strongest control response was automatically selected and used for the analysis (largest negative amplitude for FP and highest average z score for MUA). In papers II and III, the mean of all the channels of the evoked-FP and z score in each animal was used in the analysis.

In each study (papers I-III), the interval of interest (IOI) for statistical analysis was determined automatically and separately for nociceptive/tactile stimuli. The response onset was determined as the time point when the z score of the median control responses (obtained by pooled mean from all the animals) was larger than zero in the right-tailed sign test ($P<0.05$ for at least 80 ms). In papers I and II, the offsets of the nociceptive and tactile stimuli were defined at 500 ms and 60 ms after the onset, respectively. In paper III, it was empirically set to 770 ms.

Spontaneous activity (papers I and II)

Neuronal activity

Spontaneous neuronal activity was obtained per channel as for the evoked MUA, but also adding a stimulation artefact masking in the wideband signal. The average firing rate in each condition was obtained by dividing the number of spikes in all channels by the duration of the recording.

Electrocorticography

ECoG signals, sampled at 100 Hz and bandpass filtered (0.5-45 Hz), were cleaned from high-amplitude artefacts using wavelet denoising [200] and, after being divided into 5 s epochs, their power spectral densities (PSDs) were estimated and averaged.

Motor behaviour (paper II)

The rats´ speed and time presence in different zone positions in an open field were monitored by detecting and smoothening the centre of the rats´ bodies. Five-sec average epochs were used for statistical comparisons. Heat maps with single-pixel resolution were used to visualise the mean animal presence in each pixel.

Histology (papers II-IV)

HDBS biocompatibility

Quantitative analysis of tissue reactions was done at 6 (paper II) or 11-12 (paper III) weeks PS. Each marker-positive area was calculated from two sections, about 300 μm distant from the microelectrode cluster tip, as the area with an intensity above a set intensity threshold (the same for all the animals). The innermost region of interest (ROI) was defined as the area extending 50 μm from the electrode cluster, subtracting the theoretical area occupied by the electrodes. Other analyzed ROIs were 0-50, 50-100 and 100-150 μm from the innermost ROI. A reference area outside of these ROIs was used as the control. The percentage of the fluorescent area with intensity above the threshold with respect to the total ROI area was then calculated.

Nanoparticle-coated needles

Quantitative analysis of tissue responses was done at 3 or 7 days PS. The analysed sections were 4-500 μm from the cortical surface. The ROIs were defined as 0-50 μm and 50-100 μm from the border of the implanted needle. For CD68, CX3CR-1^{GFP} and GFAP markers, the same technique for intensity detection above the threshold as for the HDBS studies was used. The fraction between the fluorescent area with intensity above the threshold and the total ROI area was then calculated. The NeuN- and DAPI-positive cells were manually counted and then divided by the total ROI area.

Summary of results

Analgesia elicited by high-definition brain stimulation in PAG/DRN

Placement and microelectrode configuration (papers I-III)

For all the animals with a verified placement of the stimulation probe within or nearby the PAG/DRN (figure 7a), it was possible to identify microelectrodes which, on stimulation (with 50 Hz), inhibited nociceptive withdrawal reflexes without noticeable side effects (figure 7b). After testing various current intensities and combinations of candidate microelectrodes, it was possible to select a microelectrode subgroup and stimulation intensity which almost entirely abolished withdrawal reflexes without noticeable side effects.

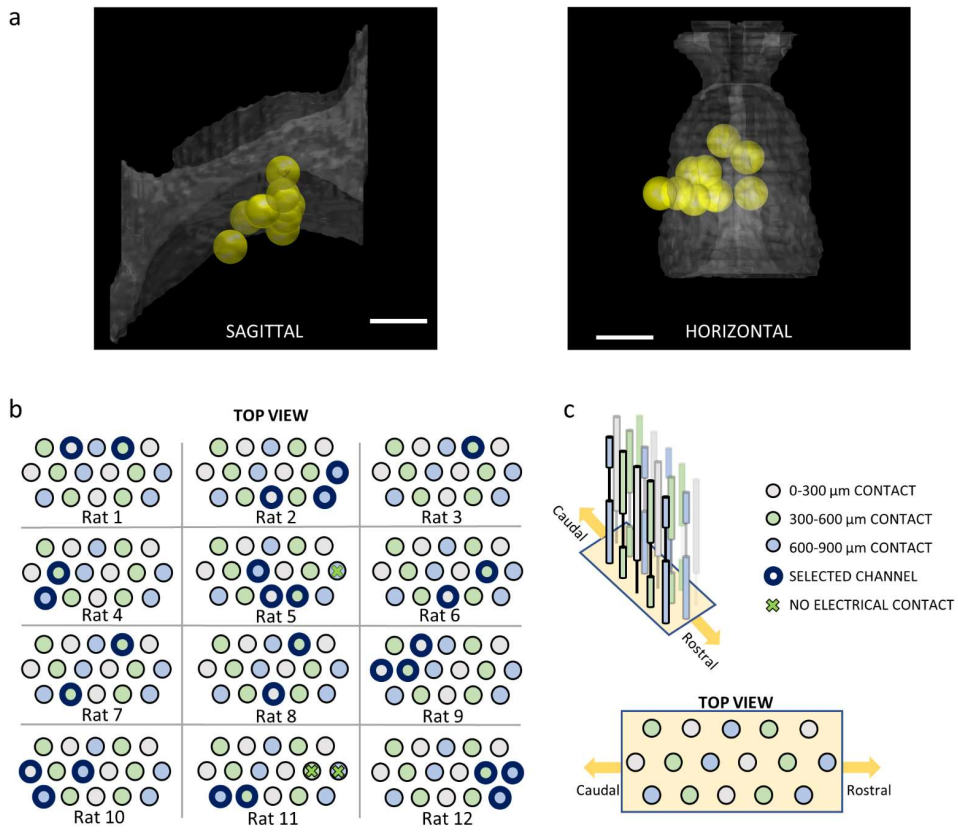

Figure 7. Stimulation microelectrode cluster placement, selection, and configuration.
(**a**) Placement of the HDBS probes in PAG/DRN in different animals (paper II). The grey area delimits the PAG/DRN borders while the yellow dots represent the distal tips of the cluster and their diameter is the averaged spread of the cluster (~700 µm). Scale bar: 1 mm. (**b**) Individually selected microelectrodes which were eliciting reflex-analgesia (representative results from paper II). (**c**) Arrangement of the microelectrodes with different contact depths within the cluster.

A summary of the median number of selected microelectrodes and current intensities per microelectrode in different papers can be found in Table 6.

Table 6. Selected stimulation configuration in different studies.
The number of selected microelectrodes and current intensity indicates the median across the animals (the total number of implanted microelectrodes per animal was 16). The area of interest represents where the CO_2-laser nociceptive-evoked stimuli were delivered.

	Selected microelectrodes (n)	Selected current intensity per microelectrode (µA)	Area of Interest
Paper I	4	40	All the four paws
Paper II	2	30	Right hind paw
Paper III	4	50	Right hind paw

As mentioned in Methods, the microelectrode contacts were displaced in a three-dimensional space within PAG/DRN (figure 7c). The selected microelectrode contacts were located in different depths in most cases (figure 7b and c). Interestingly, even though the microelectrodes cluster was found in different areas of PAG/DRN or nearby it, it was possible to find, in each rat, a subgroup of reflex-analgesic microelectrodes that did not produce side effects. This indicates that a rather large area in PAG/DRN is concerned with pain control and can be addressed by the 3D cluster technique.

Selection of stimulation parameters (paper I)

After the selection of a subgroup of microelectrodes, the analgesic efficacy of different parameters of HDBS was evaluated. To assess the magnitude of the analgesia, nociceptive-evoked cortical signals in S1, involved in the sensory-discriminative aspects of pain, were monitored. These signals are known to resemble the ones measured in humans [26, 27]. Classical nociceptive-evoked withdrawal reflex tests were also used.

Frequency

Different stimulation frequencies were tested at $I_{max.}$, defined as the maximal current per microelectrode that was possible to deliver (within acceptable current intensities of 50 μA) without inducing side effects. When using 130 or 90 Hz, aversive events, such as flight reactions or signs of increased alertness, were elicited. When observing such signs, the stimulation was discontinued immediately. Frequencies equal to or below 50 Hz did not show any noticeable side effects. Of these frequencies (5, 20 and 50 Hz) the most efficient frequency in blocking the nociceptive-evoked cortical signals and withdrawal reflexes was 50 Hz, which caused an almost total block of the nociceptive-evoked cortical signal. Frequencies of 20 and 5 Hz also produced a less robust analgesic effect (figure 8).

Intensity

When reducing the current from I_{max} of 10 or 20 μA, the analgesic effect gradually decreased. However, even if smaller in comparison to I_{max}, low stimulation intensities (10-30 μA) induced a significant reduction in the nociceptive-evoked responses (figure 8).

Selected parameters

The most efficient stimulation parameters which abolished pain-related signals among the tested ones were 50 Hz and I_{max} and they were used in the following experiments. However, it is important to note that lower stimulation frequencies and intensities might be useful in situations where a partial reduction of nociceptive input is sufficient.

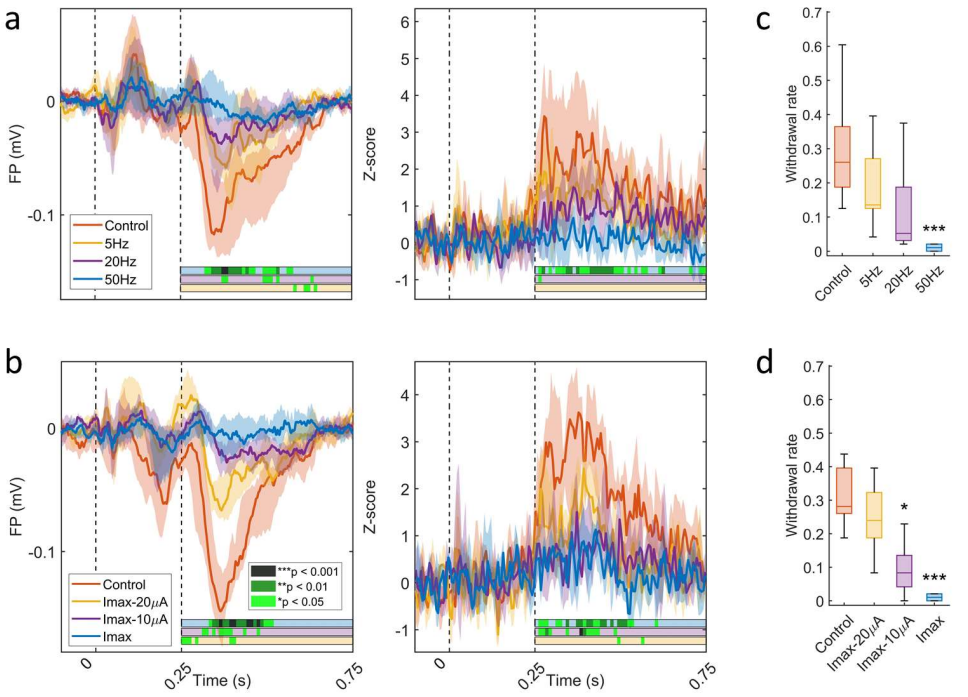

Figure 8. Selection of parameters for HDBS based on frequency- or current-dependent effects on nociceptive-evoked cortical responses in S1 and withdrawal reflexes.

(**a-b**) The coloured lines indicate the median of nociceptive-evoked field potential (left) and z score (multiunit activity; right) with fixed current (I_{max}) and varying frequency (**a**) or fixed current (50 Hz) and varying current (**b**). The shaded area around the lines represents the interquartile range and time=0 is the nociceptive stimulus onset. The bars below the graphs indicate the statistical difference between the signal amplitudes in different conditions in comparison to the control (n=8, Friedman's test with Dunn-Sidák post hoc, bin size: 10 ms). (**c-d**) Box plots (median and quartiles) of the withdrawal rate in the corresponding experimental settings of (**a**) and (**b**), respectively (n=8, Friedman's test with Dunn-Sidák post hoc). The figure shows the results obtained by nociceptive stimulations delivered on the hind limb contralateral to the implanted intracortical recording array in S1. Similar results were obtained from the forepaw.

Assessment of analgesic potency (paper I)

To benchmark its analgesic potency, the effect of HDBS on nociceptive-evoked responses was compared to the effect of morphine injections (1 mg/kg and ~3 mg/kg subcutaneously). The morphine-induced analgesia was significantly less efficient in inhibiting the nociceptive-evoked signals in S1 and withdrawal reflexes (figure 9). Importantly, while HDBS did not provoke any noticeable side effects, the rats injected with morphine showed obvious signs of sedation. These results indicate that the analgesia elicited by HDBS is superior to that of morphine.

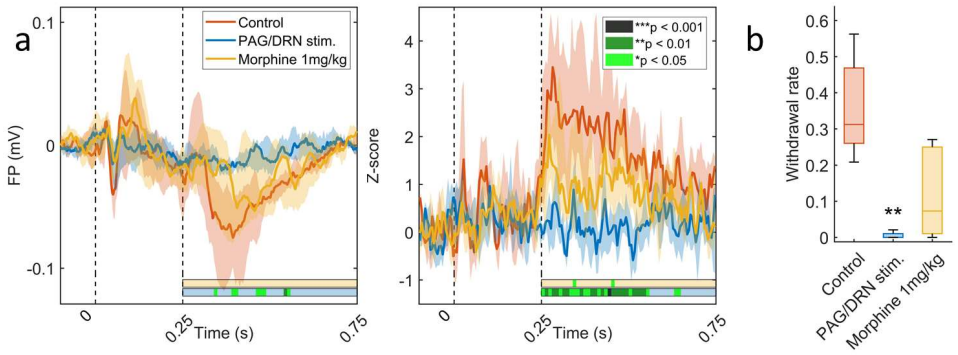

Figure 9. HDBS and morphine effect on nociceptive-evoked responses.
Graphics and statistics as in figure 8. The figure shows the results obtained by nociceptive stimulations delivered on the hind limb contralateral to the implanted intracortical recording array in S1. Similar results were obtained from the forepaw.

HDBS effect during hyperalgesia (papers I and III)

Effect on primary and secondary hyperalgesia and on altered gait during hyperalgesia

To clarify whether HDBS is effective in inhibiting pain also in sensitized conditions, its effect was tested during hyperalgesia, which is a common feature of several chronic pain conditions. The UVB-induced hyperalgesia was used as a translational model of sensitized pain. For both primary and secondary hyperalgesia areas (figure 10a), the nociceptive-evoked cortical responses recorded from S1 were found significantly and similarly reduced during HDBS (figure 10b). Similar results were found for withdrawal reflexes.

In addition, while HDBS itself did not significantly affect normal gait, it almost normalised the gait asymmetry induced by the hind paw inflammation (figure 10c).

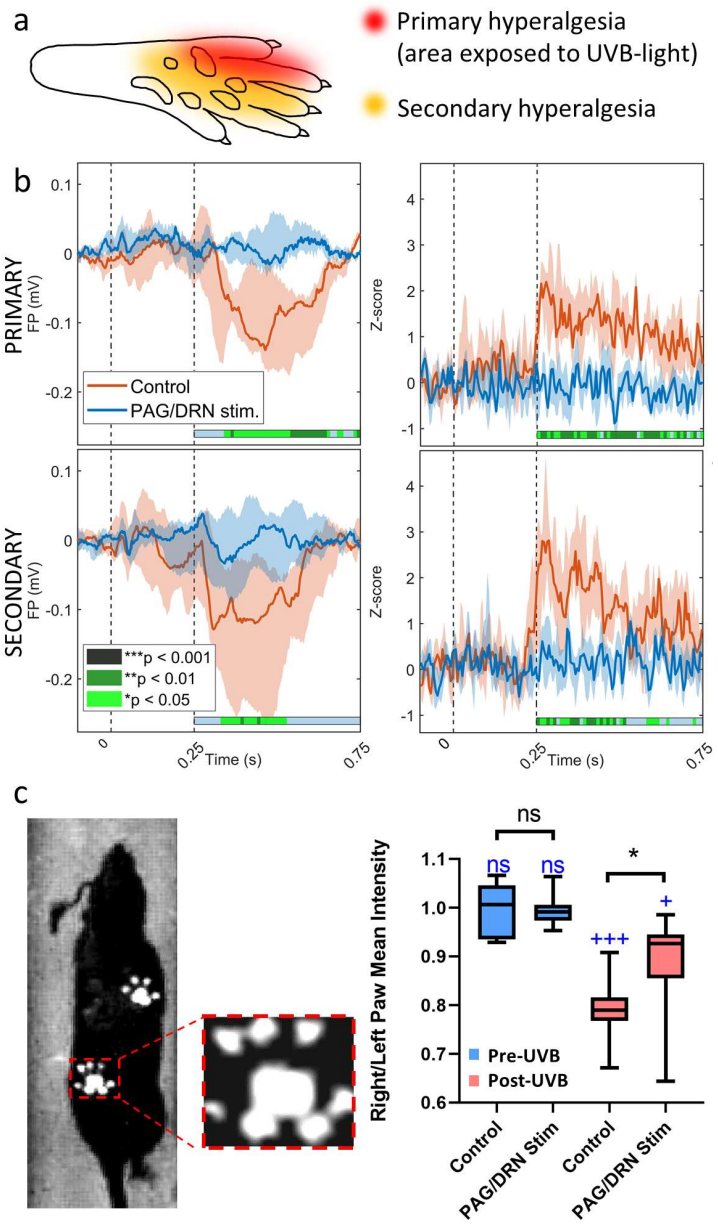

Figure 10. Effect of HDBS during hyperalgesia.
(**a**) Schematic of the paw inflammation. (**b**) The effect of HDBS on nociceptive-evoked cortical responses in S1 in primary (top) and secondary (bottom) hyperalgesia areas. Graphics as in figure 8 (n = 8, Wilcoxon matched-pairs signed-rank). (**c**) Example of the detected rat paw print from the Catwalk and its magnification (left) and box plot (whiskers and quartile; right) of the paw intensity ratio with or without HDBS in normal conditions and 2 days after UVB-exposure (n = 7, ns in blue, +++ $p < 0.001$, + $p < 0.05$, one-sample t-test; n=7, * $p < 0.05$, ns in black, Wilcoxon matched-pairs signed-rank test).

The effect on different components of pain

As previously mentioned, pain is a complex experience which includes sensory-discriminative and affective-emotional components. Several studies report that S1 is involved in the sensory-discriminative aspect of pain [24], while the ACC is assumed to be involved in the affective motivational aspect [29, 30].

In paper III, the nociceptive-evoked cortical signals with or without HDBS were monitored from both S1 and ACC to investigate whether HDBS in PAG/DRN was equally effective on the sensory and affective components of pain. The results showed that the nociceptive-evoked signals (inverted amplitude, -A, for FPs and averaged z score for MUA) were inhibited by HDBS to a similar extent, also when the experiment was repeated 2 days after UVB-induced hyperalgesia (figure 11).

Given the previous results on motor responses, it thus appears that HDBS in PAG/DRN strongly affects all major components of pain.

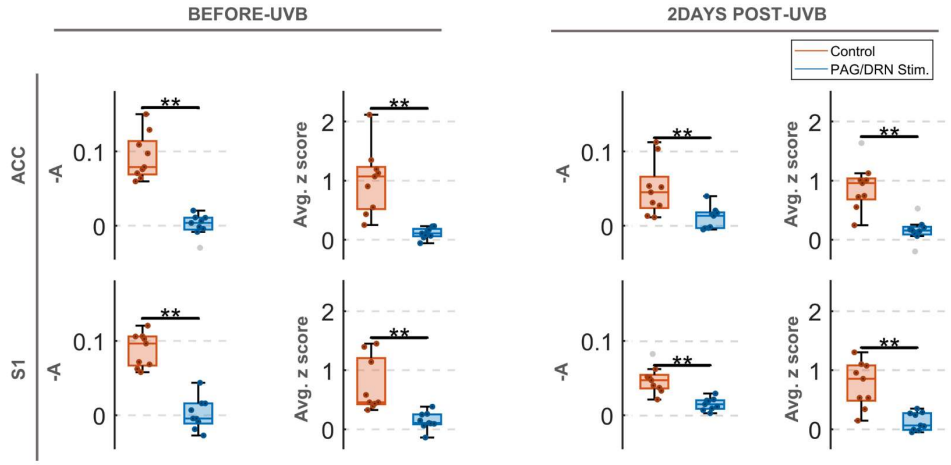

Figure 11. HDBS inhibition of cortical signals in S1 and ACC before and during hyperalgesia.
Box plot (median and quartiles) of the inverted FP response at the timepoint of maximal control response (left), and the averaged z score of MUA within the IOI (right), calculated from recordings in S1 and ACC before and during UVB-induced hyperalgesia. The coloured dots indicate the values of single animals (n=9, ** $p < 0.01$, Wilcoxon matched-pairs signed-rank). -A, inverted amplitude; Avg. z-score, averaged z score in the IOI.

Sustained analgesia during continuous HDBS (paper II)

Both in preclinical and clinical research, observations of decay in analgesic effects during continuous PAG/DRN stimulation have been reported. However, this phenomenon and whether it can be avoided has not been systematically investigated.

Four hours of continuous stimulation with constant currents at a minimal current intensity (30 µA) for blocking cortical nociceptive signals were initially completed

to observe the extent and timeline of the decay. The analgesic effect of HDBS during the stimulation time was assessed by monitoring the nociceptive-evoked cortical responses in S1 and withdrawal reflexes. A decay of effect started to be detectable between 30-60 minutes for all the evoked responses. However, while the MUA (averaged z score) and withdrawal reflexes were back to the control level after the stimulation period, the FPs (-A) were still reduced (about 50%; figure 12). The experiment was then repeated by introducing adjustments of current in steps of 5 μA when at least 3/16 nociceptive-evoked withdrawal responses were observed. With this adaptable stimulation, it was possible to obtain a sustained level of almost complete analgesia for four hours (figure 12). A post-stimulation analgesic effect was also observed. After 30 minutes, MUA and withdrawal reflexes were back to the control level, while the FPs were still significantly reduced (figure 12).

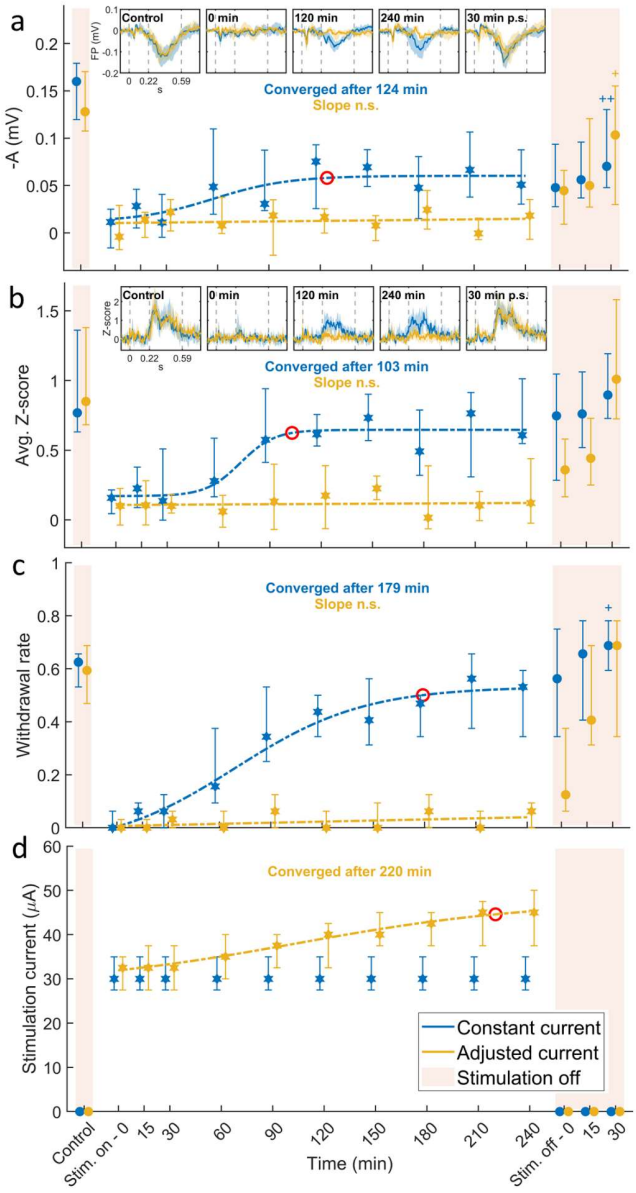

Figure 12. HDBS analgesic effect during continuous stimulation.
(**a-d**) The yellow and blue dots/stars represent the median of the nociceptive-evoked (**a**) inverted FP responses at the timepoint of maximal control response, (**b**) averaged z score of MUA within the IOI, (**c**) the withdrawal reflex rate, and (**d**) the stimulation current (n=12). The whiskers represent the interquartile range while the dashed lines indicate the sigmoid function or the linear function fitted to constant or adjusted current stimulation mode, respectively. For the sigmoid function, the red dot indicates the convergence point. The data are shown before, during and after HDBS. The latest time point (30 minutes post-HDBS) was tested against control (n=12, + $p < 0.05$, ++ $p < 0.01$, Wilcoxon matched-pairs signed-rank). The five graphs on top of (**a**) and (**b**) represent the nociceptive-evoked FP and z score at selected time points (graphics as in figure 8). −A, inverted amplitude; n.s., not significant; Avg. Z-score, averaged score in the IOI.

Reliability of HDBS across different time points (papers I-III)

Prior to the perfusion of the animals, HDBS stimulations were performed with the same microelectrode configuration and stimulation intensity as selected at the beginning of the study. This was done to clarify if the results obtained in the beginning of the studies were reproducible. These experiments were done at different time points in different studies (paper I: 10-11 weeks PS; paper II: 7 weeks PS; paper III: 11-12 weeks PS), and they all showed similar and consistent results of HDBS inhibition of nociceptive-evoked responses (figure 13). These results thus confirm reproducible effects of HDBS up to 12 weeks.

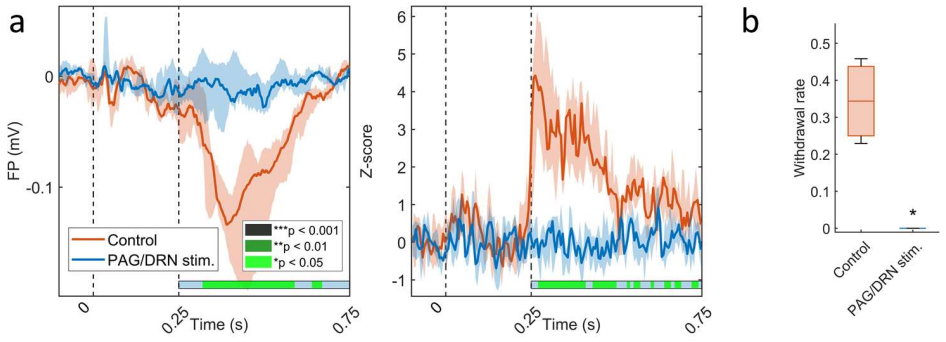

Figure 13. Verification of HDBS analgesic effect after several weeks PS.
Graphics and statistics as in figure 10 (representative results from paper I).

Investigation of side effects induced by HDBS (papers I and II)

One major challenge in developing effective treatments for chronic pain is to avoid concomitant adverse side effects. PAG/DRN is an important centre for pain control but is also involved in other physiological functions. Consequently, stimulation-produced side effects are commonly reported in both animal [55, 142] and human [201-203] studies. In agreement with the literature, stimulation-triggered side effects such as movements of the head, flight reactions, rotations, urination, increased breathing, and signs of alertness [52, 55, 136] were commonly observed during the selection phase of HDBS. Given the clinical value of avoiding side effects, it was of great importance to further confirm the lack of side effects during HDBS. These experiments included monitoring the spontaneous cortical activity (MUA and ECoG), effects on behaviour, and tactile input to S1.

Spontaneous activity and brain states

Despite the large number of studies on PAG, the effects of its stimulation on cortical spontaneous activity have not been investigated before in awake and freely moving animals but it is an essential piece of information which would clarify the functional role of PAG/DRN. In addition, information about the effects of PAG/DRN stimulation on brain states is essential to assess its clinical potential.

With that in mind, the effects of PAG/DRN stimulation on spontaneous MUA were recorded by intracortical microelectrodes implanted in S1. The effect of morphine (1 mg/kg subcutaneous injection) was recorded for comparison. The spontaneous MUA was not significantly affected by HDBS. On the contrary, injections of morphine significantly lowered the spontaneous firing rate (figure 14a).

The ECoG were also investigated with or without PAG/DRN stimulation to clarify potential HDBS effects on brain states. The power spectral densities were similar in control conditions and during HDBS (figure 14b) suggesting no significant adverse effects.

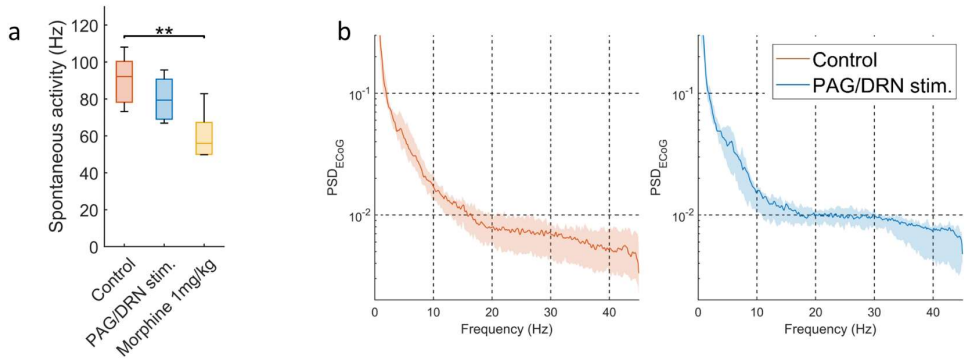

Figure 14. HDBS effects on spontaneous cortical activity.
(**a**) Box plot (median and quartiles) of the spontaneous neuronal firing rate in different conditions (n = 7, **p < 0.01, Friedman's test with Dunn-Sidák post hoc). (**b**) Power spectral densities of the ECoGs. PSD, power spectral density.

Motor activity

The rats´ movements were analysed in an environment with water, food, or nesting material in different corners of the cage (figure 15a). This was performed to clarify whether physiological functions such as hunger, thirst or nesting behaviour might be compromised by HDBS.

The analysis did not show any significant difference in the time spent in different corners (figure 15b). The average speed and the percentage of time spent moving were also not significantly changed (figure 15c). These results indicate that normal behaviour is not significantly affected by HDBS.

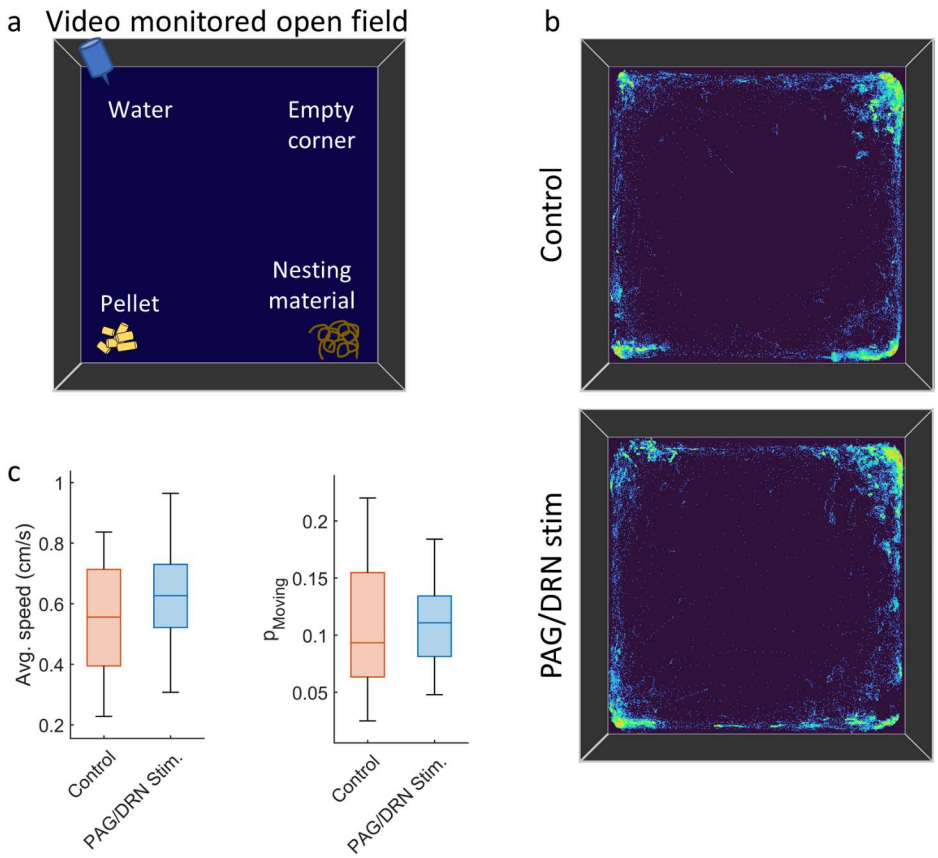

Figure 15. Analysis of HDBS effect on rat behaviour in the open field.
(**a**) Schematic of the open field used in motion tracking experiment and EcoG recordings with water, pellet and nesting material in different corners. (**b**) Heat maps of the rat presence probability (blue-to-red corresponds to low-to-high probability). (**c**) Box plot (median and quartiles) of the averaged speed (left; Avg. speed) and probability of movement (right; P_{moving}; n=11, Wilcoxon matched-pairs signed-rank).

Tactile cortical input

Despite it has been demonstrated that PAG exerts an anti-nociceptive effect by inhibiting the nociceptive inputs at the level of the spinal cord [53, 55, 57, 204, 205], it has not been previously shown whether this effect is nociception-specific or if it might affect other afferent inputs such as tactile input. In fact, many nociceptive dorsal horn neurons also receive an excitatory tactile input [206].

To clarify this aspect, the tactile-evoked responses recorded from S1 were monitored with or without HDBS. A small but significant reduction in the tactile input was found (figure 16). However, in comparison to the almost total block of nociceptive input, the tactile-input reduction was a modest effect indicating that

PAG/DRN stimulation has a high preferential effect on nociceptive afferent pathways. This finding suggests that touch sensitivity is not compromised by HDBS in PAG/DRN.

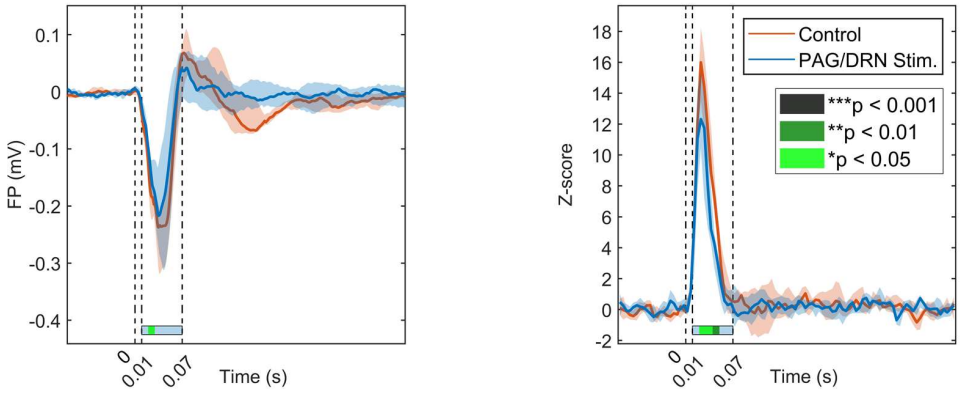

Figure 16. HDBS effect on tactile input to S1.
Graphics and statistics as in figure 10. The figure shows the results obtained by tactile stimulations delivered on the hind limb contralateral to the implanted intracortical recording array in S1. Similar results were obtained from the forepaw.

Neural tissue reactions to implants

HDBS probe (papers II and III)

To assess the biocompatibility of the implanted microelectrodes, a histological analysis was done in order to quantify the tissue reactions to the implanted electrodes. The percentage of ED1-positive area was significantly different from controls only in the area between 0-50 µm from the implanted microelectrodes (figure 17a and c). The percentage of GFAP-positive area was significantly different from controls between 0-100 µm (figure 17b and d). The quantification of the neuronal death indicated a slight, but not significant reduction of neurons from 0 to 50 µm from the implanted microelectrodes. Together, these results indicate a very small impact of the implanted microelectrodes on the neural tissue and thus a high degree of biocompatibility.

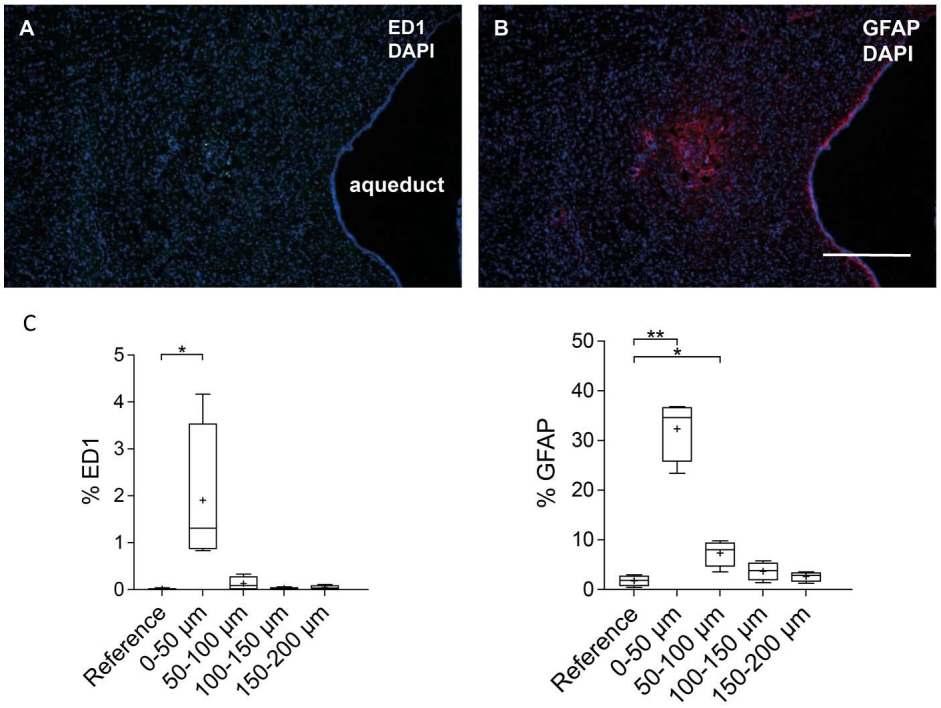

Figure 17. Tissue response to the implanted stimulation microelectrodes.
(**A-B**) Representative immunofluorescent pictures of horizontal tissue slices showing activated microglia (ED1, in green; **A**), or activated astrocytes (GFAP, in red; **B**) and cell nuclei (DAPI, in blue; **A-B**). Scale bar: 500 µm. (**C**) Box plot (median and quartiles) of the percentage of fluorescent area for ED1 and GFAP. The mean is also indicated with a +. The analysed areas are 0-50 µm, 50-100 µm, 100-150 µm and 150-200 µm from the implanted probe (n=4, *p < 0.05, **p < 0.01, Friedman test with Dunn's multiple comparisons).

Coated needles with minocycline-loaded nanoparticles (paper IV)

As mentioned in Methods, minocycline is an antibiotic with neuroprotective and anti-inflammatory properties [192, 193]. In addition, it has been demonstrated that PLGA-nanoparticles loaded with this drug could sustain its release for over 30 days [90]. To verify whether the inflammatory reaction due to implants can be reduced, stainless steel needles were coated with gelatine incorporating minocycline-loaded PLGA nanoparticles and implanted in the cortex of mice for local and sustained drug release. Control needles coated with gelatine without nanoparticles were also implanted. Histological analysis showed a significant reduction of the activated astrocytes (GFAP) and microglia (CD68) 7 days after the implantation and a significant reduction of activated astrocytes 3 days after the implantation in comparison to controls (figure 18).

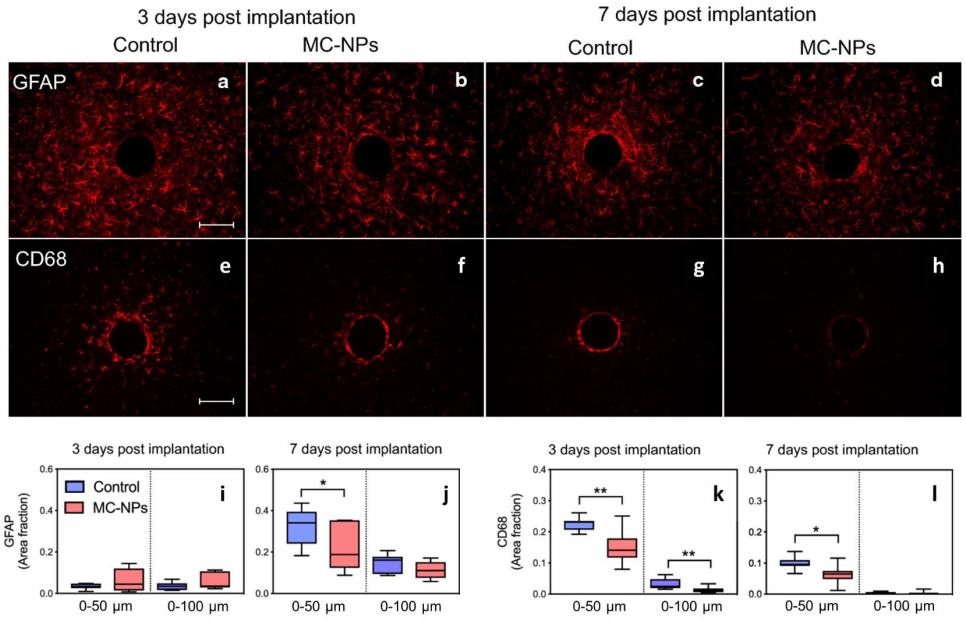

Figure 18. Reduction of tissue responses induced by minocycline-loaded nanoparticles.
(**a-h**) Representative immunofluorescent pictures of horizontal tissue slices showing activated astrocytes (GFAP; top), or activated microglia (CD68; bottom). Scale bar: 100 µm. (**i-l**) Box plot (median and quartiles) of the fraction of fluorescent area for GFAP and CD68. The analysed areas are 0-50 µm and 50-100 µm (* $p < 0.05$, ** $p < 0.01$, Mann–Whitney test).

These results suggest that gelatine coatings of electrical-neural interfaces incorporating minocycline-loaded nanoparticles could be useful to reduce the inflammatory response.

Discussion

The experimental and developmental work in this thesis was motivated by the lack of satisfactory treatments for numerous patients with persistent pain. To exploit the analgesic power of the PAG/DRN system, a new approach to achieve high-definition brain stimulation was developed and evaluated. This approach includes a critical step of selecting an appropriate subset from a surplus of implanted microelectrodes in PAG/DRN to increase the analgesia/side effect ratio. In addition, to clarify HDBS analgesic effect in normal and hyperalgesia conditions, several clinically relevant aspects were also evaluated (such as durability of analgesia, reliability, specificity, and biocompatibility) as well as the presence of side effects.

To assess the pain and analgesia most likely felt in awake animals, recordings were made in cortical areas involved in sensory-discriminative and affective aspects of pain in addition to motor assessments (reflexes, gait, and movement tracking during natural behaviours). Translational techniques were used to elicit pure pain and hyperalgesia to increase the clinical relevance of the results.

The highlights of this thesis can be summarised as follows:

- A novel implantable microelectrode array enabling a granular deep brain stimulation pattern in three dimensions was developed.
- HDBS in PAG/DRN enabled sustained, powerful, and selective analgesia in normal and hyperalgesia conditions without noticeable adverse side effects.
- A novel animal model of pain and analgesia, providing information on sensory-discriminative, affective, and motor aspects of pain, was developed to assess the potential of HDBS.
- The microelectrode cluster techniques used for HDBS elicited minimal tissue reactions which is a necessary requirement for a sustainable therapy.

On the developed HDBS technology

Modulation of neural networks by introducing artificial electrical fields is known to be a powerful tool to treat a variety of diseases or to attenuate their symptoms [207-

211]. Commercially available probes for brain stimulation are based on relatively large contacts (diameter of ~1.5 mm) located around the circumference of a single cylindrical shaft [126, 129]. Despite their successful use in a variety of disorders, this design has substantial limitations. For instance, the widespread stimulation fields are not ideal when employed in complex areas such as the PAG. The poor spatial resolution of conventional DBS electrodes may be a major reason for the inability to avoid adverse side effects and has therefore likely hampered previous attempts to exploit the analgesic potential of this area [139-141]. In addition, tissue reactions and loss of nearby neurons caused by their relatively large size might have significantly contributed to the decay of analgesic effects reported after a few weeks from the implantation in PAG [212-214].

The use of flexible microelectrodes has been shown to provoke fewer tissue reactions in comparison to large and stiff electrodes [86, 172, 173]. However, due to their small surface area, only small currents can be injected without exceeding safety levels [86, 215]. Therefore, they need to be precisely placed. Thus, differences in anatomy between individuals or unintended misplacements become a challenge. To overcome these problems, the idea behind the technique for high-definition brain stimulation used in the thesis is the implantation of a surplus of separated microelectrodes and the subsequent selection of a subgroup of appropriate microelectrodes. The results of this thesis support the concept that side effects can be eliminated by an individualized selection of microelectrodes and, consequently, an appropriate selection of stimulation sites.

Stimulation via the microelectrodes excluded during the selection phase often elicited side effects indicating that alternative pathways could be elicited by stimulating areas just nearby the selected reflex-analgesic microelectrodes. Side effects could also be induced by the selected microelectrodes by using a too-high stimulation intensity indicating that specificity is reached only when fine-tuning a granular stimulation field. These results provide clear evidence for the notion that side effects are to a large degree site-specific, as previously suggested by other studies and attempts of PAG mapping [52, 64]. They also confirm the presence of a complex neural network in this area in which pain control is a part of a larger behavioural control [16, 37, 40]. For instance, it is known that PAG is involved in the control of several autonomic functions and adapted behaviour in stressful/threatening situations. This finding also supports the hypothesis that the unsatisfactory results achieved using conventional DBS electrodes are dependent on their low stimulation specificity causing a mix of analgesia and unpleasant side effects.

Opioids, such as morphine, are considered the strongest analgesic. However, their effect is unreliable and often connected to serious side effects with a strong impact on patient lives such as sedation [69, 70]. HDBS not only showed a more potent effect but was not provoking sedation. Moreover, despite the extensive search for side effects using a battery of tests including quantitative movement analysis,

analysis of ECoG, and spontaneous cortical signalling, assessment of tactile input to S1, no adverse side effects were discovered.

Notably, side effects and reliability are to this date the major problem in most of the known methods to inhibit pain (see “Current pain therapies” in Introduction), especially in chronic conditions [216]. Thus, the finding that HDBS in PAG/DRN enabled potent pain inhibition without adverse side effects in all the tested individuals is very promising.

Biocompatibility

The high degree of biocompatibility of this method for brain stimulation is supported by the results of minimal tissue reactions and the stability of stimulation intensities thresholds across several weeks. These findings are consistent with and extend previous reports indicating high biocompatibility of ultra-flexible microelectrodes and gelatine vehicles [157, 172, 217].

The non-significant loss of neurons is also relevant to minimize the stimulation current needed to activate neurons and reach therapeutic effects. This in turn reduces the risk of the current spread to sites which cause side effects. In addition, as already mentioned, high biocompatibility is likely to reduce the risk of therapeutic failure or reduced efficacy over time (an observation which is commonly reported in the clinic) [147, 212, 213, 218]. Hence, these features indicate that HDBS has the potential to fulfil the safety requirements and the ultimate goal of a sustainable therapy in clinical settings.

The findings that incorporating minocycline nanoparticles in gelatine locally reduces tissue inflammation induced by the implantation trauma might be used to further minimise the impact of invasive techniques on the neural tissue.

The potential for applications outside PAG/DRN

The technique for high-resolution brain stimulation holds the potential to be adapted and used in brain areas with different architecture and structural organization for the treatment of various disorders. For instance, HDBS has been demonstrated to successfully reduce motor symptoms in a rodent model of Parkinson's disease [176].

The design of the probe can be adapted to fit different neural structures and stimulation needs or individual differences. For example, the size of the deinsulated contact area, their depth, the microelectrode spread and number, and the wire arrangement can be easily tailored. Moreover, also the type of stimulation, the stimulation parameters, and the number of combinations can be adjusted depending on the brain area, the disorder, and the individual.

HDBS could be also used for mapping and investigating the function of various brain circuits. In this thesis, for example, the combination of HDBS and cortical recordings has elucidated the effect of PAG/DRN stimulation on both sensory discriminative and affective nociceptive pathways to the cortex, which has not been accomplished before.

On pain assessment in animals

Since pain is a subjective experience, a perception, that can only exist in a conscious state, valid assessments of pain and analgesia need to be made in awake individuals. Such assessments in awake freely moving animals have previously relied mainly on motor responses such as nociceptive withdrawal reflexes or other behavioural responses [54, 55, 219]. However, many studies suggest a dissociation between nociceptive motor responses and the pain sensation [27, 105]. In addition, behavioural responses can be misleading in several situations since they do not represent a direct measure of perceived pain. To assess pain in animals, this thesis made use of and further developed a novel animal model of pain based on cortical recordings of nociceptive signals [27]. It is known that evoked potentials in or nearby S1 recorded with EEG methods correlate strongly to the magnitude of perceived pain in humans, e.g., to the magnitude of pain [24, 26]. By recording analogous signals in S1 in awake and unrestrained rodents, in addition to just reflex or behavioural responses, a more valid animal model for pain and analgesia was thus achieved. In the third investigation in this thesis, this model was, for the first time, expanded to also include cortical regions that in humans have been linked to the affective aspects of pain to simultaneously assess all major aspects of pain in the same individual. Hence, the developed techniques provide a much more valid and direct readout of pain most likely felt by the animals than previous models of pain.

Notably, previous animal studies on the analgesic effects of PAG/DRN in awake animals did not take into consideration that PAG/DRN might exert a differential effect on the nociceptive pathways involved in the different aspects of pain (such as sensory, affective, or motor components).

The results from our studies indicate that PAG/DRN modulates the sensory-discriminative and affective-related nociceptive pathways and nociceptive reflexes in a similar way. However, a dissociation appeared over prolonged HDBS where the inhibition of withdrawal reflexes was less robust in comparison to inhibition of the cortical signals. This may suggest that PAG/DRN can exert a differential effect on the three aspects of pain in order to, for example, preserve acute defensive reactions in critical situations.

Importantly, the same methodology using stable cortical long-term recordings in awake freely moving animals allowed: i) evaluation of long-term changes during

alteration of the pain system (for example, induction of hyperalgesia); ii) assessment of the stimulation impact on, e.g., tactile input to S1; iii) investigation of changes in brain states through the ECoG. The amount of information obtained from this animal model is thus enormous. In addition, control data can be obtained from the respective animals reducing the number of animals needed in the experiments.

On the sustainability of HDBS and future directions

Even though the presented technique for brain stimulation was demonstrated to be efficacious in inhibiting pain, the prolonged PAG/DRN stimulation needed small step (5 μA) increases in current intensity (total of 5-15 μA) to maintain a complete block of nociceptive transmission to the cortex for 4 hours. The fact that an incremental increase in current could recover the initial effect may suggest that a decrease in neural excitability is induced by the stimulation. Alternative mechanisms might be fatigue or plastic changes at the level of PAG/DRN or in the descending connections. Regardless of the precise mechanism, strategies to decrease the metabolic load on the stimulated neurons should be investigated to prevent this decay of effect over time. One possibility is to make use of the present results indicating that analgesia can be induced from a wide area within PAG/DRN (paper II). This is further supported by the finding that the contacts of the selected electrodes often were located at different depths in the same individual. Therefore, a stimulation probe with a wider spread and possibly including more electrodes than used here might be designed and used to find multiple analgesic sub-clusters which can be used in alternation. Such a probe might also be utilised to create maps of stimulation-produced effects within the same animal which would improve the knowledge of the somatotopic organisation in PAG/DRN. This, in turn, may provide a basis for further optimisation of the cluster design. Other strategies to combat decay may include the use of lower frequencies than 50 Hz or the use of intermittent stimulation, given the finding of a post-stimulation analgesic effect. In this perspective, an important finding was that the nociceptive-evoked responses could be almost totally inhibited using the same stimulation parameters and microelectrodes in many sessions for up to 11-12 weeks. This shows that, if allowed to recover, the system will restore the initial analgesic effects. A prerequisite for this is likely the high degree of biocompatibility revealed by the histological analysis. Hence, introducing pauses in the stimulation could be a viable option for chronic pain treatment.

In the current work, the selection of the electrode subset was done by combining microelectrode stimulation based on their individual analgesic effect. However, this search lacks the synergistic effect that might arise from the stimulation of multiple microelectrodes and just a few of the possible combinations were tested. Algorithms which can predict the optimal combination based on the multiple activation of a

group of microelectrodes might take into consideration this synergistic effect and might also be faster. Notably, in humans, cortical recordings will not be necessary, since the patient can directly score the pain intensity allowing a much faster and more accurate process than is possible in animal studies.

In this thesis, the effect of PAG/DRN was tested on acute pain and during hyperalgesia, which is a persistent but relatively short-lasting (days) change of the nociceptive system. To enable future clinical trials, evaluations of HDBS efficacy on animal models of more prolonged persistent pain conditions may be needed.

Finally, an animal model composed of long-term neuronal recordings and a system, such as HDBS in PAG/DRN, to turn on/off nociception on demand might be useful also to define signatures or biomarkers of different chronic pain disorders (even at the individual level), which is a major challenge of the pain field. These biomarkers could be used, for example, to evaluate the potency of new pain therapies in animal studies.

Conclusions

The overall results of this thesis show that HDBS in PAG/DRN can elicit a potent, specific, sustained, reliable analgesic effect in normal conditions and during hyperalgesia with minimal side effects and high biocompatibility. This suggests that HDBS in PAG/DRN holds the potential to treat persistent pain by selectively and reliably inhibiting the transmission of nociceptive signals to cortical areas related to discriminative and affective aspects of pain. However, more studies are needed, in particular on chronic pain, to further clarify how to accomplish a truly sustainable pain treatment.

The work presented here shows that a novel approach for brain stimulation based on microwires spread in the target tissue followed by a new stimulation paradigm comprising a selection of the implanted microelectrodes might be efficient and reliable as well as safe for pain treatments. This approach might be the base for a new way of thinking for new generations of brain stimulation probes and improved therapies for a variety of disorders. Furthermore, simultaneous monitoring of nociceptive-evoked responses in different areas of the brain might be a valid pain-assessment model to obtain precise information about the nociceptive processing as well as the perceived pain in animals. Finally, coatings containing drug-loaded nanoparticles might be useful to further improve the biocompatibility of therapies based on brain implants.

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References

1. Global Burden of Disease Study, C., *Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013.* Lancet, 2015. **386**(9995): p. 743-800.
2. Gustavsson, A., et al., *Socio-economic burden of patients with a diagnosis related to chronic pain--register data of 840,000 Swedish patients.* Eur J Pain, 2012. **16**(2): p. 289-99.
3. Phillips, C.J., *The Cost and Burden of Chronic Pain.* Rev Pain, 2009. **3**(1): p. 2-5.
4. Gilam, G., et al., *What Is the Relationship between Pain and Emotion? Bridging Constructs and Communities.* Neuron, 2020. **107**(1): p. 17-21.
5. Calati, R., et al., *The impact of physical pain on suicidal thoughts and behaviors: Meta-analyses.* Journal of Psychiatric Research, 2015. **71**: p. 16-32.
6. Daneshjou, K., H. Jafarieh, and S.R. Raaeskarami, *Congenital Insensitivity to Pain and Anhydrosis (CIPA) Syndrome; A Report of 4 Cases.* Iran J Pediatr, 2012. **22**(3): p. 412-6.
7. Pain, I.A.f.t.S.o. *IASP Announces Revised Definition of Pain*. 2020; Available from: https://www.iasp-pain.org/publications/iasp-news/iasp-announces-revised-definition-of-pain/.
8. Rainville, P., et al., *Pain affect encoded in human anterior cingulate but not somatosensory cortex.* Science, 1997. **277**(5328): p. 968-971.
9. Kulkarni, B., et al., *Attention to pain localization and unpleasantness discriminates the functions of the medial and lateral pain systems.* European Journal of Neuroscience, 2005. **21**(11): p. 3133-3142.
10. Talbot, K., et al., *The sensory and affective components of pain: are they differentially modifiable dimensions or inseparable aspects of a unitary experience? A systematic review.* British Journal of Anaesthesia, 2019. **123**(2): p. E263-E272.
11. Bajwa, Z.H., B.L. Wilsey, and S.M. Fishman, *Anatomy and Physiology of Pain*, in *Office Practice of Neurology*, M.A. Samuels and S.K. Feske, Editors. 2003, Churchill Livingstone. p. 1383-1389.
12. Treede, R.D., et al., *The cortical representation of pain.* Pain, 1999. **79**(2-3): p. 105-11.
13. Tobimatsu, S., *Understanding cortical pain perception in humans.* Neurology and Clinical Neuroscience, 2021. **9**(1): p. 24-29.
14. Bingel, U., et al., *Subcortical structures involved in pain processing: evidence from single-trial fMRI.* Pain, 2002. **99**(1-2): p. 313-21.

15. Schouenborg, J., *Action-based sensory encoding in spinal sensorimotor circuits.* Brain Res Rev, 2008. **57**(1): p. 111-7.
16. Benarroch, E.E., *Periaqueductal gray: an interface for behavioral control.* Neurology, 2012. **78**(3): p. 210-7.
17. Løseth, G.E., D.M. Ellingson, and S. Leknes. *Touch and pain*. Noba textbook series: Psychology 2022; Available from: https://nobaproject.com/modules/touch-and-pain.
18. Melzack, R., *Pain and the neuromatrix in the brain.* J Dent Educ, 2001. **65**(12): p. 1378-82.
19. Melzack, R., *Evolution of the neuromatrix theory of pain. The Prithvi Raj Lecture: presented at the third World Congress of World Institute of Pain, Barcelona 2004.* Pain Pract, 2005. **5**(2): p. 85-94.
20. Kucyi, A. and K.D. Davis, *The dynamic pain connectome.* Trends Neurosci, 2015. **38**(2): p. 86-95.
21. Umeda, T., T. Isa, and Y. Nishimura, *The somatosensory cortex receives information about motor output.* Science Advances, 2019. **5**(7).
22. Borich, M.R., et al., *Understanding the role of the primary somatosensory cortex: Opportunities for rehabilitation.* Neuropsychologia, 2015. **79**: p. 246-255.
23. Catani, M., *A little man of some importance.* Brain, 2017. **140**: p. 3055-3061.
24. Vierck, C.J., et al., *Role of primary somatosensory cortex in the coding of pain.* Pain, 2013. **154**(3): p. 334-344.
25. Coghill, R.C., et al., *Pain intensity processing within the human brain: A bilateral, distributed mechanism.* Journal of Neurophysiology, 1999. **82**(4): p. 1934-1943.
26. Jin, Q.Q., et al., *Somatotopic Representation of Second Pain in the Primary Somatosensory Cortex of Humans and Rodents.* J Neurosci, 2018. **38**(24): p. 5538-5550.
27. Ljungquist, B., et al., *Discrepancies between cortical and behavioural long-term readouts of hyperalgesia in awake freely moving rats.* European Journal of Pain, 2016. **20**(10): p. 1689-1699.
28. Stevens, F.L., R.A. Hurley, and K.H. Taber, *Anterior Cingulate Cortex: Unique Role in Cognition and Emotion.* Journal of Neuropsychiatry and Clinical Neurosciences, 2011. **23**(2): p. 120-125.
29. Wager, T.D., et al., *Pain in the ACC?* Proc Natl Acad Sci U S A, 2016. **113**(18): p. E2474-5.
30. Fuchs, P.N., et al., *The anterior cingulate cortex and pain processing.* Front Integr Neurosci, 2014. **8**: p. 35.
31. Xiao, X. and Y.Q. Zhang, *A new perspective on the anterior cingulate cortex and affective pain.* Neurosci Biobehav Rev, 2018. **90**: p. 200-211.
32. Gungor, N.Z. and J. Johansen, *A Chronic Pain in the ACC.* Neuron, 2019. **102**(5): p. 903-905.
33. Hurt, R.W. and H.T. Ballantine, Jr., *Stereotactic anterior cingulate lesions for persistent pain: a report on 68 cases.* Clin Neurosurg, 1974. **21**: p. 334-51.

34. Johansen, J.P. and H.L. Fields, *Glutamatergic activation of anterior cingulate cortex produces an aversive teaching signal.* Nat Neurosci, 2004. **7**(4): p. 398-403.
35. Mercer Lindsay, N., et al., *Brain circuits for pain and its treatment.* Sci Transl Med, 2021. **13**(619): p. eabj7360.
36. Carrillo, M., et al., *Emotional Mirror Neurons in the Rat's Anterior Cingulate Cortex.* Current Biology, 2019. **29**(8): p. 1301-+.
37. Behbehani, M.M., *Functional characteristics of the midbrain periaqueductal gray.* Prog Neurobiol, 1995. **46**(6): p. 575-605.
38. Depaulis, A. and R. Bandler, *The Midbrain Periaqueductal Gray Matter Functional, Anatomical, and Neurochemical Organization.* 1991: Springer.
39. Carrive, P., *The periaqueductal gray and defensive behavior: functional representation and neuronal organization.* Behav Brain Res, 1993. **58**(1-2): p. 27-47.
40. Carrive, P. and M.M. Morgan, *Periaqueductal Gray*, in *The Human Nervous System* J.K. Mai and G. Paxinos, Editors. 2012, Academic Press. p. 367-400.
41. Bandler, R. and M.T. Shipley, *Columnar organization in the midbrain periaqueductal gray: modules for emotional expression?* Trends Neurosci, 1994. **17**(9): p. 379-89.
42. Hornung, J.P., *The human raphe nuclei and the serotonergic system.* J Chem Neuroanat, 2003. **26**(4): p. 331-43.
43. Monti, J.M., *The structure of the dorsal raphe nucleus and its relevance to the regulation of sleep and wakefulness.* Sleep Med Rev, 2010. **14**(5): p. 307-17.
44. Dolen, G., et al., *Social reward requires coordinated activity of nucleus accumbens oxytocin and serotonin.* Nature, 2013. **501**(7466): p. 179-84.
45. Niederkofler, V., et al., *Identification of Serotonergic Neuronal Modules that Affect Aggressive Behavior.* Cell Rep, 2016. **17**(8): p. 1934-1949.
46. Li, Y., et al., *Serotonin neurons in the dorsal raphe nucleus encode reward signals.* Nat Commun, 2016. **7**: p. 10503.
47. Cohen, J.Y., M.W. Amoroso, and N. Uchida, *Serotonergic neurons signal reward and punishment on multiple timescales.* Elife, 2015. **4**.
48. Wang, Q.P. and Y. Nakai, *The dorsal raphe: an important nucleus in pain modulation.* Brain Res Bull, 1994. **34**(6): p. 575-85.
49. Cai, Y.Q., et al., *Optogenetic activation of brainstem serotonergic neurons induces persistent pain sensitization.* Mol Pain, 2014. **10**: p. 70.
50. Keay, K.A. and R. Bandler, *Periaqueductal Gray*, in *The Rat Nervous System* G. Paxinos, Editor. 2015, Academic Press. p. 243-257.
51. Hosobuchi, Y., J.E. Adams, and R. Linchitz, *Pain relief by electrical stimulation of the central gray matter in humans and its reversal by naloxone.* Science, 1977. **197**(4299): p. 183-6.
52. Fardin, V., J.L. Oliveras, and J.M. Besson, *A reinvestigation of the analgesic effects induced by stimulation of the periaqueductal gray matter in the rat. I. The production of behavioral side effects together with analgesia.* Brain Res, 1984. **306**(1-2): p. 105-23.

53. Behbehani, M.M. and H.L. Fields, *Evidence that an excitatory connection between the periaqueductal gray and nucleus raphe magnus mediates stimulation produced analgesia.* Brain Res, 1979. **170**(1): p. 85-93.
54. Mayer, D.J. and J.C. Liebeskind, *Pain reduction by focal electrical stimulation of the brain: an anatomical and behavioral analysis.* Brain Res, 1974. **68**(1): p. 73-93.
55. Oliveras, J.L., et al., *Behavioral and electrophysiological evidence of pain inhibition from midbrain stimulation in the cat.* Exp Brain Res, 1974. **20**(1): p. 32-44.
56. Basbaum, A.I. and H.L. Fields, *Endogenous pain control mechanisms: review and hypothesis.* Ann Neurol, 1978. **4**(5): p. 451-62.
57. Basbaum, A.I. and H.L. Fields, *The origin of descending pathways in the dorsolateral funiculus of the spinal cord of the cat and rat: further studies on the anatomy of pain modulation.* J Comp Neurol, 1979. **187**(3): p. 513-31.
58. Zhang, D.X., C.M. Owens, and W.D. Willis, *Two forms of inhibition of spinothalamic tract neurons produced by stimulation of the periaqueductal gray and the cerebral cortex.* J Neurophysiol, 1991. **65**(6): p. 1567-79.
59. Ossipov, M.H., G.O. Dussor, and F. Porreca, *Central modulation of pain.* J Clin Invest, 2010. **120**(11): p. 3779-87.
60. Lovick, T.A., *The periaqueductal gray-rostral medulla connection in the defence reaction: efferent pathways and descending control mechanisms.* Behav Brain Res, 1993. **58**(1-2): p. 19-25.
61. Ennis, M., et al., *Projections from the periaqueductal gray to the rostromedial pericoerulear region and nucleus locus coeruleus: anatomic and physiologic studies.* J Comp Neurol, 1991. **306**(3): p. 480-94.
62. Keay, K.A. and R. Bandler, *Parallel circuits mediating distinct emotional coping reactions to different types of stress.* Neuroscience and Biobehavioral Reviews, 2001. **25**(7-8): p. 669-678.
63. Bandler, R., et al., *Central circuits mediating patterned autonomic activity during active vs. passive emotional coping.* Brain Research Bulletin, 2000. **53**(1): p. 95-104.
64. Soper, W.Y. and R. Melzack, *Stimulation-produced analgesia: evidence for somatotopic organization in the midbrain.* Brain Res, 1982. **251**(2): p. 301-11.
65. Mokhtar, M. and P. Singh, *Neuroanatomy, Periaqueductal Gray*, in *StatPearls*. 2021: Treasure Island (FL).
66. Fong, A. and S.A. Schug, *Pathophysiology of pain: a practical primer.* Plast Reconstr Surg, 2014. **134**(4 Suppl 2): p. 8S-14S.
67. Raja, S.N., R.A. Meyer, and J.N. Campbell, *Hyperalgesia and sensitization of primary afferent fibers*, in *Pain Syndromes in Neurology*, H.L. Fields, Editor. 1990, Butterworth-Heinemann. p. 19-45.
68. Portenoy, R.K., *Current pharmacotherapy of chronic pain.* Journal of Pain and Symptom Management, 2000. **19**(1): p. S16-S20.
69. Dhingra, L., et al., *Cognitive Effects and Sedation.* Pain Med, 2015. **16 Suppl 1**: p. S37-43.

70. Shaiova, L., *The management of opioid-related sedation.* Curr Pain Headache Rep, 2005. **9**(4): p. 239-42.
71. Angst, M.S., et al., *Pain sensitivity and opioid analgesia: A pharmacogenomic twin study.* Pain, 2012. **153**(7): p. 1397-1409.
72. Reuben, S.S., *A new class of COX-2 inhibitors offer an alternative to NSAIDS in pain management after spinal surgery.* Spine (Phila Pa 1976), 2001. **26**(13): p. 1505-6.
73. Machado, G.C., et al., *Non-steroidal anti-inflammatory drugs (NSAIDs) for musculoskeletal pain.* BMJ, 2021. **372**: p. n104.
74. Kiefer, W. and G. Dannhardt, *COX-2 inhibition and pain management: a review summary.* Expert Rev Clin Immunol, 2005. **1**(3): p. 431-42.
75. Scott, N.A., et al., *Trigger Point Injections for Chronic Non-Malignant Musculoskeletal Pain: A Systematic Review.* Pain Medicine, 2009. **10**(1): p. 54-69.
76. Klein, M.M., et al., *Transcranial magnetic stimulation of the brain: guidelines for pain treatment research.* Pain, 2015. **156**(9): p. 1601-1614.
77. Bhatia, G., et al., *Intrathecal Drug Delivery (ITDD) systems for cancer pain.* F1000Res, 2013. **2**: p. 96.
78. Delhaas, E.M. and F. Huygen, *Complications associated with intrathecal drug delivery systems.* BJA Educ, 2020. **20**(2): p. 51-57.
79. Harber, V.J. and J.R. Sutton, *Endorphins and exercise.* Sports Med, 1984. **1**(2): p. 154-71.
80. Hassed, C., *Mind-body therapies--use in chronic pain management.* Aust Fam Physician, 2013. **42**(3): p. 112-7.
81. Schmelz, M., et al., *Nerve growth factor antibody for the treatment of osteoarthritis pain and chronic low-back pain: mechanism of action in the context of efficacy and safety.* Pain, 2019. **160**(10): p. 2210-2220.
82. Dib-Hajj, S.D., P. Geha, and S.G. Waxman, *Sodium channels in pain disorders: pathophysiology and prospects for treatment.* Pain, 2017. **158**(4): p. S97-S107.
83. King, A., *Analgesia without opioids.* Nature, 2019. **573**: p. S4-S6.
84. Zhang, M.D., et al., *Ca2+- binding protein NECAB2 facilitates inflammatory pain hypersensitivity.* Journal of Clinical Investigation, 2018. **128**(9): p. 3757-3768.
85. Che, T., et al., *Biased ligands at opioid receptors: Current status and future directions.* Science Signaling, 2021. **14**(677).
86. McCreery, D., et al., *Microelectrode array for chronic deep-brain microstimulation and recording.* IEEE Trans Biomed Eng, 2006. **53**(4): p. 726-37.
87. Steigerwald, F., et al., *Directional deep brain stimulation of the subthalamic nucleus: A pilot study using a novel neurostimulation device.* Movement Disorders, 2016. **31**(8): p. 1240-1243.
88. Aonuma, S., et al., *A high-resolution computational localization method for transcranial magnetic stimulation mapping.* Neuroimage, 2018. **172**: p. 85-93.
89. Bagshaw, K.R., et al., *Pain management via local anesthetics and responsive hydrogels.* Ther Deliv, 2015. **6**(2): p. 165-76.

90. Holmkvist, A.D., et al., *Hydrophobic ion pairing of a minocycline/Ca(2+)/AOT complex for preparation of drug-loaded PLGA nanoparticles with improved sustained release.* Int J Pharm, 2016. **499**(1-2): p. 351-357.
91. Braz, J.M., et al., *Forebrain GABAergic Neuron Precursors Integrate into Adult Spinal Cord and Reduce Injury-Induced Neuropathic Pain.* Neuron, 2012. **74**(4): p. 663-675.
92. Oren, O. and J. Ablin, *Precision medicine: the emerging approach to the chronic pain patient.* International Journal of Clinical Rheumatology, 2013. **8**(4).
93. Diaz, M.M., et al., *Toward Composite Pain Biomarkers of Neuropathic Pain-Focus on Peripheral Neuropathic Pain.* Front Pain Res (Lausanne), 2022. **3**: p. 869215.
94. Mao, J., *Current challenges in translational pain research.* Trends Pharmacol Sci, 2012. **33**(11): p. 568-73.
95. Yekkirala, A.S., et al., *Breaking barriers to novel analgesic drug development.* Nat Rev Drug Discov, 2017. **16**(11): p. 810.
96. Woolf, C.J., *Overcoming obstacles to developing new analgesics.* Nat Med, 2010. **16**(11): p. 1241-7.
97. Mogil, J.S., *The translatability of pain across species.* Philosophical Transactions of the Royal Society B-Biological Sciences, 2019. **374**(1785).
98. Granmo, M., T. Jensen, and J. Schouenborg, *Nociceptive Transmission to Rat Primary Somatosensory Cortex - Comparison of Sedative and Analgesic Effects.* Plos One, 2013. **8**(1).
99. Mogil, J.S., K.D. Davis, and S.W. Derbyshire, *The necessity of animal models in pain research.* Pain, 2010. **151**(1): p. 12-17.
100. Mogil, J.S., *Animal models of pain: progress and challenges.* Nature Reviews Neuroscience, 2009. **10**(4): p. 283-294.
101. Bishop, T., et al., *Characterisation of ultraviolet-B-induced inflammation as a model of hyperalgesia in the rat.* Pain, 2007. **131**(1-2): p. 70-82.
102. Barrot, M., *Tests and Models of Nociception and Pain in Rodents.* Neuroscience, 2012. **211**: p. 39-50.
103. Chapman, C.R., et al., *Pain measurement: an overview.* Pain, 1985. **22**(1): p. 1-31.
104. Gregory, N.S., et al., *An overview of animal models of pain: disease models and outcome measures.* J Pain, 2013. **14**(11): p. 1255-69.
105. Sandrini, G., et al., *The lower limb flexion reflex in humans.* Progress in Neurobiology, 2005. **77**(6): p. 353-395.
106. Dolgin, E., *Animalgesic effects.* Nature Medicine, 2010. **16**(11): p. 1237-1240.
107. Ponsel, S., et al., *Alterations of distributed neuronal network oscillations during acute pain in freely-moving mice.* IBRO Rep, 2020. **9**: p. 195-206.
108. LeBlanc, B.W., et al., *Cortical theta is increased while thalamocortical coherence is decreased in rat models of acute and chronic pain.* Pain, 2014. **155**(4): p. 773-782.
109. LeBlanc, B.W., et al., *Electroencephalographic signatures of pain and analgesia in rats.* Pain, 2016. **157**(10): p. 2330-2340.

110. Fu, B., et al., *Acute and chronic pain affects local field potential of the medial prefrontal cortex in different band neural oscillations.* Molecular Pain, 2018. **14**.
111. Zhang, Z.G., et al., *Gamma-Band Oscillations in the Primary Somatosensory Cortex-A Direct and Obligatory Correlate of Subjective Pain Intensity.* Journal of Neuroscience, 2012. **32**(22): p. 7429-7438.
112. Peng, W.W., et al., *Brain oscillations reflecting pain-related behavior in freely moving rats.* Pain, 2018. **159**(1): p. 106-118.
113. Iannetti, G.D., et al., *Determinants of laser-evoked EEG responses: Pain perception or stimulus saliency?* Journal of Neurophysiology, 2008. **100**(2): p. 815-828.
114. Iannetti, G.D. and A. Mouraux, *From the neuromatrix to the pain matrix (and back).* Experimental Brain Research, 2010. **205**(1): p. 1-12.
115. Tsoucalas, G. and M. Sgantzos, *Electric current to cure arthritis and cephalaea in ancient Greek medicine.* Mediterrenean Journal of Rheumatology, 2016. **27**(4): p. 198-203.
116. Kellaway, P., *The part played by electric fish in the early history of bioelectricity and electrotherapy.* Bull Hist Med, 1946. **20**(2): p. 112-37.
117. Caputi, F., et al., *Luigi Rolando and his pioneering efforts to relate structure to function in the nervous system.* J Neurosurg, 1995. **83**(5): p. 933-7.
118. Pearce, J.M., *Marie-Jean-Pierre Flourens (1794-1867) and cortical localization.* Eur Neurol, 2009. **61**(5): p. 311-4.
119. Shampo, M.A., R.A. Kyle, and D.P. Steensma, *Walter Hess-Nobel Prize for work on the brain.* Mayo Clin Proc, 2011. **86**(10): p. E49.
120. Heath, R.G., *Electrical Self-Stimulation of the Brain in Man.* Am J Psychiatry, 1963. **120**: p. 571-7.
121. Delgado, J.M.R., *Regulating man. Physical control of the mind. Toward a psychocivilised society*. World perspectives. 1969: Harper and Row. 280.
122. Aum, D.J. and T.S. Tierney, *Deep brain stimulation: foundations and future trends.* Front Biosci (Landmark Ed), 2018. **23**(1): p. 162-182.
123. Spiegel, E.A., et al., *Stereotaxic Apparatus for Operations on the Human Brain.* Science, 1947. **106**(2754): p. 349-350.
124. Pool, J.L., *Psychosurgery in older people.* J Am Geriatr Soc, 1954. **2**(7): p. 456-66.
125. Delgado, J.M.R., et al., *Intracerebral Radio Stimulation and Recording in Completely Free Patients.* Journal of Nervous and Mental Disease, 1968. **147**(4): p. 329-&.
126. Krauss, J.K., et al., *Technology of deep brain stimulation: current status and future directions.* Nat Rev Neurol, 2021. **17**(2): p. 75-87.
127. van Kuyck, K., et al., *Histological alterations induced by electrode implantation and electrical stimulation in the human brain: a review.* Neuromodulation, 2007. **10**(3): p. 244-61.
128. Anderson, D.N., et al., *The muDBS: Multiresolution, Directional Deep Brain Stimulation for Improved Targeting of Small Diameter Fibers.* Front Neurosci, 2019. **13**: p. 1152.

129. Steigerwald, F., C. Matthies, and J. Volkmann, *Directional Deep Brain Stimulation.* Neurotherapeutics, 2019. **16**(1): p. 100-104.
130. Schnitzler, A., et al., *Directional Deep Brain Stimulation for Parkinson's Disease: Results of an International Crossover Study With Randomized, Double-Blind Primary Endpoint.* Neuromodulation, 2022. **25**(6): p. 817-828.
131. Rojas, E., et al., *A comparison of an implanted accelerometer with a wearable accelerometer for closed-loop DBS.* Annu Int Conf IEEE Eng Med Biol Soc, 2022. **2022**: p. 3439-3442.
132. Meidahl, A.C., et al., *Adaptive Deep Brain Stimulation for Movement Disorders: The Long Road to Clinical Therapy.* Mov Disord, 2017. **32**(6): p. 810-819.
133. Bouthour, W., et al., *Biomarkers for closed-loop deep brain stimulation in Parkinson disease and beyond.* Nat Rev Neurol, 2019. **15**(6): p. 343-352.
134. Dembek, T.A., et al., *Sweetspot Mapping in Deep Brain Stimulation: Strengths and Limitations of Current Approaches.* Neuromodulation, 2022. **25**(6): p. 877-887.
135. Reynolds, D.V., *Surgery in the rat during electrical analgesia induced by focal brain stimulation.* Science, 1969. **164**(3878): p. 444-5.
136. Liebeskind, J.C., et al., *Analgesia from electrical stimulation of the periaqueductal gray matter in the cat: behavioral observations and inhibitory effects on spinal cord interneurons.* Brain Res, 1973. **50**(2): p. 441-6.
137. Richardson, D.E. and H. Akil, *Pain Reduction by Electrical Brain-Stimulation in Man .1. Acute Administration in Periaqueductal and Periventricular Sites.* Journal of Neurosurgery, 1977. **47**(2): p. 178-183.
138. Bittar, R.G., et al., *Deep brain stimulation for pain relief: a meta-analysis.* J Clin Neurosci, 2005. **12**(5): p. 515-9.
139. Levy, R., T.R. Deer, and J. Henderson, *Intracranial neurostimulation for pain control: a review.* Pain Physician, 2010. **13**(2): p. 157-65.
140. Farrell, S.M., A. Green, and T. Aziz, *The Current State of Deep Brain Stimulation for Chronic Pain and Its Context in Other Forms of Neuromodulation.* Brain Sci, 2018. **8**(8).
141. Boccard, S.G., E.A. Pereira, and T.Z. Aziz, *Deep brain stimulation for chronic pain.* J Clin Neurosci, 2015. **22**(10): p. 1537-43.
142. Fardin, V., J.L. Oliveras, and J.M. Besson, *A reinvestigation of the analgesic effects induced by stimulation of the periaqueductal gray matter in the rat. II. Differential characteristics of the analgesia induced by ventral and dorsal PAG stimulation.* Brain Res, 1984. **306**(1-2): p. 125-39.
143. Besson, J.M., V. Fardin, and J.L. Oliveras, *Analgesia Produced by Stimulation of the Periaqueductal Gray-Matter - True Antinoceptive Effects Versus Stress Effects.* Midbrain Periaqueductal Gray Matter, 1991. **213**: p. 121-138.
144. Del-Ben, C.M. and F.G. Graeff, *Panic disorder: is the PAG involved?* Neural Plast, 2009. **2009**: p. 108135.
145. Hyam, J.A., et al., *The autonomic effects of deep brain stimulation--a therapeutic opportunity.* Nat Rev Neurol, 2012. **8**(7): p. 391-400.
146. Lovick, T., *Deep brain stimulation and autonomic control.* Exp Physiol, 2014. **99**(2): p. 320-5.

147. Pereira, E.A. and T.Z. Aziz, *Neuropathic pain and deep brain stimulation.* Neurotherapeutics, 2014. **11**(3): p. 496-507.
148. Nielsen, M.S., et al., *Chronic subthalamic high-frequency deep brain stimulation in Parkinson's disease--a histopathological study.* Eur J Neurol, 2007. **14**(2): p. 132-8.
149. Buzsaki, G., C.A. Anastassiou, and C. Koch, *The origin of extracellular fields and currents--EEG, ECoG, LFP and spikes.* Nat Rev Neurosci, 2012. **13**(6): p. 407-20.
150. Hong, G.S. and C.M. Lieber, *Novel electrode technologies for neural recordings (vol 20, pg 330, 2019).* Nature Reviews Neuroscience, 2019. **20**(6): p. 376-376.
151. Wellman, S.M. and T.D.Y. Kozai, *Understanding the Inflammatory Tissue Reaction to Brain Implants To Improve Neurochemical Sensing Performance.* Acs Chemical Neuroscience, 2017. **8**(12): p. 2578-2582.
152. Lecomte, A., E. Descamps, and C. Bergaud, *A review on mechanical considerations for chronically-implanted neural probes.* J Neural Eng, 2018. **15**(3): p. 031001.
153. Kozai, T.D., et al., *Brain tissue responses to neural implants impact signal sensitivity and intervention strategies.* ACS Chem Neurosci, 2015. **6**(1): p. 48-67.
154. Kumosa, L.S., V. Zetterberg, and J. Schouenborg, *Gelatin promotes rapid restoration of the blood brain barrier after acute brain injury.* Acta Biomater, 2018. **65**: p. 137-149.
155. Bjornsson, C.S., et al., *Effects of insertion conditions on tissue strain and vascular damage during neuroprosthetic device insertion.* J Neural Eng, 2006. **3**(3): p. 196-207.
156. Polikov, V.S., P.A. Tresco, and W.M. Reichert, *Response of brain tissue to chronically implanted neural electrodes.* Journal of Neuroscience Methods, 2005. **148**(1): p. 1-18.
157. Kohler, P., et al., *Influence of probe flexibility and gelatin embedding on neuronal density and glial responses to brain implants.* PLoS One, 2015. **10**(3): p. e0119340.
158. Gilletti, A. and J. Muthuswamy, *Brain micromotion around implants in the rodent somatosensory cortex.* J Neural Eng, 2006. **3**(3): p. 189-95.
159. Biran, R., D.C. Martin, and P.A. Tresco, *The brain tissue response to implanted silicon microelectrode arrays is increased when the device is tethered to the skull.* J Biomed Mater Res A, 2007. **82**(1): p. 169-78.
160. Wellman, S.M., F. Cambi, and T.D.Y. Kozai, *The role of oligodendrocytes and their progenitors on neural interface technology: A novel perspective on tissue regeneration and repair.* Biomaterials, 2018. **183**: p. 200-217.
161. Kettenmann, H. and B.R. Ransom, *Neuroglia*. Third ed. 2012: Oxford University Press. 601.
162. Kozai, T.D., et al., *In vivo two-photon microscopy reveals immediate microglial reaction to implantation of microelectrode through extension of processes.* J Neural Eng, 2012. **9**(6): p. 066001.
163. Corraliza, I., *Recruiting specialized macrophages across the borders to restore brain functions.* Front Cell Neurosci, 2014. **8**: p. 262.

164. Szarowski, D.H., et al., *Brain responses to micro-machined silicon devices.* Brain Res, 2003. **983**(1-2): p. 23-35.
165. Fujita, T., et al., *Cellular dynamics of macrophages and microglial cells in reaction to stab wounds in rat cerebral cortex.* Acta Neurochir (Wien), 1998. **140**(3): p. 275-9.
166. Lee, H.H., et al., *Time Course and Characteristics of Astrocyte Activation in the Rat Brain after Injury.* Korean J Neurotrauma, 2015. **11**(2): p. 44-51.
167. Campbell, A. and C. Wu, *Chronically Implanted Intracranial Electrodes: Tissue Reaction and Electrical Changes.* Micromachines (Basel), 2018. **9**(9).
168. Landis, D.M., *The early reactions of non-neuronal cells to brain injury.* Annu Rev Neurosci, 1994. **17**: p. 133-51.
169. Venero, J.L., et al., *The executioners sing a new song: killer caspases activate microglia.* Cell Death Differ, 2011. **18**(11): p. 1679-91.
170. McConnell, G.C., et al., *Implanted neural electrodes cause chronic, local inflammation that is correlated with local neurodegeneration.* Journal of Neural Engineering, 2009. **6**(5).
171. Yong, H.Y.F., et al., *The benefits of neuroinflammation for the repair of the injured central nervous system.* Cellular & Molecular Immunology, 2019. **16**(6): p. 540-546.
172. Luan, L., et al., *Ultraflexible nanoelectronic probes form reliable, glial scar-free neural integration.* Sci Adv, 2017. **3**(2): p. e1601966.
173. Stice, P., et al., *Thin microelectrodes reduce GFAP expression in the implant site in rodent somatosensory cortex.* J Neural Eng, 2007. **4**(2): p. 42-53.
174. Edell, D.J., et al., *Factors influencing the biocompatibility of insertable silicon microshafts in cerebral cortex.* IEEE Trans Biomed Eng, 1992. **39**(6): p. 635-43.
175. Kozai, T.D.Y., et al., *Reduction of neurovascular damage resulting from microelectrode insertion into the cerebral cortex using in vivo two-photon mapping.* Journal of Neural Engineering, 2010. **7**(4).
176. Mohammed, M., et al., *Ice coating -A new method of brain device insertion to mitigate acute injuries.* J Neurosci Methods, 2020. **343**: p. 108842.
177. Wei, X., et al., *Nanofabricated Ultraflexible Electrode Arrays for High-Density Intracortical Recording.* Adv Sci (Weinh), 2018. **5**(6): p. 1700625.
178. Mercanzini, A., et al., *Demonstration of cortical recording using novel flexible polymer neural probes.* Sensors and Actuators a-Physical, 2008. **143**(1): p. 90-96.
179. Lind, G., C.E. Linsmeier, and J. Schouenborg, *The density difference between tissue and neural probes is a key factor for glial scarring.* Scientific Reports, 2013. **3**.
180. Thelin, J., et al., *Implant size and fixation mode strongly influence tissue reactions in the CNS.* PLoS One, 2011. **6**(1): p. e16267.
181. Kim, Y.T., et al., *Chronic response of adult rat brain tissue to implants anchored to the skull.* Biomaterials, 2004. **25**(12): p. 2229-37.
182. Kipke, D.R., D.S. Pellinen, and R.J. Vetter, *Advanced neural implants using thin-film polymers.* 2002 Ieee International Symposium on Circuits and Systems, Vol Iv, Proceedings, 2002: p. 173-176.

183. Zhao, Z.T., et al., *Parallel, minimally-invasive implantation of ultra-flexible neural electrode arrays.* Journal of Neural Engineering, 2019. **16**(3).
184. Ware, T., et al., *Thiol-ene/acrylate substrates for softening intracortical electrodes.* Journal of Biomedical Materials Research Part B-Applied Biomaterials, 2014. **102**(1): p. 1-11.
185. Ejserholm, F., et al., *A polymer neural probe with tunable flexibility.* 2013 6th International Ieee/Embs Conference on Neural Engineering (Ner), 2013: p. 691-694.
186. Capadona, J.R., et al., *Stimuli-responsive polymer nanocomposites inspired by the sea cucumber dermis.* Science, 2008. **319**(5868): p. 1370-1374.
187. Lind, G., et al., *Gelatine-embedded electrodes-a novel biocompatible vehicle allowing implantation of highly flexible microelectrodes.* Journal of Neural Engineering, 2010. **7**(4).
188. Lecomte, A., et al., *Silk and PEG as means to stiffen a parylene probe for insertion in the brain: toward a double time-scale tool for local drug delivery.* Journal of Micromechanics and Microengineering, 2015. **25**(12).
189. Jeon, M., et al., *Partially flexible MEMS neural probe composed of polyimide and sucrose gel for reducing brain damage during and after implantation.* Journal of Micromechanics and Microengineering, 2014. **24**(2).
190. Mercanzini, A., et al., *Controlled release nanoparticle-embedded coatings reduce the tissue reaction to neuroprostheses.* Journal of Controlled Release, 2010. **145**(3): p. 196-202.
191. Kim, D.H. and D.C. Martin, *Sustained release of dexamethasone from hydrophilic matrices using PLGA nanoparticles for neural drug delivery.* Biomaterials, 2006. **27**(15): p. 3031-3037.
192. Henry, C.J., et al., *Minocycline attenuates lipopolysaccharide (LPS)-induced neuroinflammation, sickness behavior, and anhedonia.* J Neuroinflammation, 2008. **5**: p. 15.
193. Abraham, J., et al., *Minocycline attenuates microglia activation and blocks the long-term epileptogenic effects of early-life seizures.* Neurobiol Dis, 2012. **46**(2): p. 425-30.
194. Canales, A., et al., *Multifunctional fibers for simultaneous optical, electrical and chemical interrogation of neural circuits in vivo.* Nature Biotechnology, 2015. **33**(3): p. 277-+.
195. Ziegler, D., T. Suzuki, and S. Takeuchi, *Fabrication of flexible neural probes with built-in microfluidic channels by thermal bonding of Parylene.* Journal of Microelectromechanical Systems, 2006. **15**(6): p. 1477-1482.
196. Mercanzini, A., et al., *Controlled release drug coatings on flexible neural probes.* Annu Int Conf IEEE Eng Med Biol Soc, 2007. **2007**: p. 6613-6.
197. Agorelius, J., *Development of highly biocompatible neuro-electronic interfaces towards monitoring authentic neuronal signaling in the brain*, in *Experimental Medical Sciences*. 2020, Lund University: Lund. p. 69.
198. Morgan, M.M. and J.C. Liebeskind, *Site specificity in the development of tolerance to stimulation-produced analgesia from the periaqueductal gray matter of the rat.* Brain Res, 1987. **425**(2): p. 356-9.

199. Wang, N., et al., *Differential modulation of electrical stimulation of periaqueductal gray and thalamus on nociceptive behaviors of rats.* Sheng Li Xue Bao, 2016. **68**(2): p. 115-25.
200. Turnip, A. and J. Pardede, *Artefacts Removal of EEG Signals with Wavelet Denoising.* 8th International Conference on Mechanical and Manufacturing Engineering 2017 (Icme'17), 2017. **135**.
201. Green, A.L., et al., *Intra-operative deep brain stimulation of the periaqueductal grey matter modulates blood pressure and heart rate variability in humans.* Neuromodulation, 2010. **13**(3): p. 174-81.
202. Jenck, F., J.L. Moreau, and J.R. Martin, *Dorsal Periaqueductal Gray-Induced Aversion as a Simulation of Panic Anxiety - Elements of Face and Predictive-Validity.* Psychiatry Research, 1995. **57**(2): p. 181-191.
203. Pereira, E.A., et al., *Ventral periaqueductal grey stimulation alters heart rate variability in humans with chronic pain.* Exp Neurol, 2010. **223**(2): p. 574-81.
204. Fields, H.L., et al., *Nucleus raphe magnus inhibition of spinal cord dorsal horn neurons.* Brain Res, 1977. **126**(3): p. 441-53.
205. Basbaum, A.I. and H.L. Fields, *Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry.* Annu Rev Neurosci, 1984. **7**: p. 309-38.
206. Koch, S.C., D. Acton, and M. Goulding, *Spinal Circuits for Touch, Pain, and Itch.* Annu Rev Physiol, 2018. **80**: p. 189-217.
207. Agrawal, S., et al., *Deep Brain Stimulation for Alzheimer Disease: Physiological Considerations and Anesthetic Challenges.* J Neurosurg Anesthesiol, 2022.
208. Figee, M., et al., *Deep Brain Stimulation for Depression.* Neurotherapeutics, 2022. **19**(4): p. 1229-1245.
209. Mosley, P.E., et al., *Deep brain stimulation for treatment-refractory obsessive-compulsive disorder: Opportunities and challenges.* Aust N Z J Psychiatry, 2022. **56**(11): p. 1523-1524.
210. Paro, M.R., et al., *Deep brain stimulation for movement disorders after stroke: a systematic review of the literature.* J Neurosurg, 2022: p. 1-14.
211. Bucur, M. and C. Papagno, *Deep Brain Stimulation in Parkinson Disease: A Meta-analysis of the Long-term Neuropsychological Outcomes.* Neuropsychol Rev, 2022.
212. Plotkin, R., *Results in 60 cases of deep brain stimulation for chronic intractable pain.* Appl Neurophysiol, 1982. **45**(1-2): p. 173-8.
213. Dieckmann, G. and A. Witzmann, *Initial and long-term results of deep brain stimulation for chronic intractable pain.* Appl Neurophysiol, 1982. **45**(1-2): p. 167-72.
214. Richardson, D.E. and H. Akil, *Long term results of periventricular gray self-stimulation.* Neurosurgery, 1977. **1**(2): p. 199-202.
215. Kuncel, A.M. and W.M. Grill, *Selection of stimulus parameters for deep brain stimulation.* Clin Neurophysiol, 2004. **115**(11): p. 2431-41.
216. Villars, P., et al., *Differences in the prevalence and severity of side effects based on type of analgesic prescription in patients with chronic cancer pain.* J Pain Symptom Manage, 2007. **33**(1): p. 67-77.

217. Etemadi, L., et al., *Embedded Ultrathin Cluster Electrodes for Long-Term Recordings in Deep Brain Centers.* PLoS One, 2016. **11**(5): p. e0155109.
218. Mayer, D.J. and R.L. Hayes, *Stimulation-produced analgesia: development of tolerance and cross-tolerance to morphine.* Science, 1975. **188**(4191): p. 941-3.
219. van Praag, H. and H. Frenk, *The development of stimulation-produced analgesia (SPA) in the rat.* Brain Res Dev Brain Res, 1991. **64**(1-2): p. 71-6.