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HEAT STIMULATION AS A MODULATORY TOOL FOR THE HISTAMINERGIC AND NON-HISTAMINERGIC ITCH

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HEAT STIMULATION AS A MODULATORY TOOL FOR THE HISTAMINERGIC AND NON-HISTAMINERGIC ITCH

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Daniele obtained his B.Sc and M.Sc in pharmaceutical biotechnology from the University of Milan (Italy).
Subsequently he worked as research assistant in Prof. Abbracchio's lab in Milan with focus on trigeminal pain and the role of glial cells in this sensory modality.
In 2016 he enrolled at Aalborg University as a PhD fellow at the Center for Neuroplasticity and Pain under the supervision of Lars Arendt-Nielsen.
During his research experiences he develop a solid scientific knowledge and expertise in the biological sciences, from a completely in-vitro approach to human preclinical studies.
He is interested in neurosciences in particular the mechanism underlying pain and itch perception as well as how these two relate with thermosensation.
He focused his research on the evaluation of the effects that various heat stimuli have on different itch modalities.
Daniele had the opportunity to present his work at different international conferences and school both as poster and oral presentation.

ENGLISH SUMMARY

Itch is a sensation characterized by the innate desire to scratch. Although acute itch is generally tolerable, chronic itch (itch occurring for more than 6 weeks) can severely affect the patients' quality of life and have a prevalence up to 25%. In the last decades, many therapies and treatments have been developed, but to date, their efficacies are scarce and may present with side effects. If efficient non-pharmacological treatment options could be developed this would have a major clinical potential.
Scratching itchy skin is ultimately a form of self-inflicted mechanical pain that can help in relieving itch to some extent. Therefore, many studies have focused on the interaction between itch and pain.
Regarding heat stimulations, it has been shown that long noxious heat stimuli can inhibit itch, but on the other hand, such stimuli may cause hyperalgesia and skin damage.
Many clinical observations indicate that patients can experience increased itch in warm environments, suggesting some relationships between heat and itch sensations.
A 70 years old study showed that a paradoxical itch sensation could be achieved when anaesthetised skin was stimulated with what was defined as a "burning" sensation.
Taken together these independent observations, seem to indicate that thermal perception may have a role in modulating different aspects of itch sensation, and therefore it is of interest to understand better the mechanism underlining these modulatory effects.

Within this context, this PhD project aimed at investing these aspects: 1) the effects of very short heat stimuli (ranging from innocuous to noxious) on various itch modalities; 2) how continuous skin warming affects itch intensity in human surrogate itch models; 3) investigate and possibly confirm with a more modern and systematic approach the observed dysesthesia of paradoxical itch sensation when a "burning" stimulus is applied on top of anaesthetised skin.
The first study showed that homotopical transient noxious heat stimuli were able to inhibit histaminergic itch and, to a lesser extent, non-histaminergic itch induced by cowhage. When the stimuli were applied heterotopically there was no significant inhibition suggesting that, at least for very transient stimuli, the observed inhibitory effect relies on peripheral mechanisms.
The second study showed that mild skin heating selectively aggravates serotoninergic and histaminergic itch but not cowhage-induced itch. These

results partially agree with what is observed in animals (increased serotoninergic itch intensity) and parts are in direct contrast with them (unchanged histaminergic itch intensity).
The third study failed to confirm what was previously in a 70 years old observation. Applying a heat ramp (ranging from warm to noxious heat) on anaesthetised skin did not evoke a paradoxical itch sensation at any of the time points analysed.
In conclusion, this dissertation unveils a potential role of thermal stimulation to modulate itch. Further studies are needed to investigate this in a clinical context.

DANSK RESUME

Kløe er en følelse, som karakteriseres ved et instinktivt ønske om at kradse sig. Selvom akut kløe generelt er udholdelig, så kan kronisk kløe (dvs. kløe som varer ved i mere end seks uger) påvirke patienternes livskvalitet alvorligt, og kronisk kløe har en prævalens på op til 25%. I de seneste årtier er der blevet søgt udviklet mange terapiformer og behandlinger til lindring af kløe, men virkningen af disse har indtil nu vist sig at være mangelfuld og forbundet med bivirkninger. Hvis der kunne udvikles ikke-farmakologiske behandlingsmuligheder til kløe, ville disse have et betydeligt klinisk potentiale. Når man kradser i kløende hud, påfører man ultimativt sig selv en slags selvforskyldt mekanisk smerte, som til en vis grad kan hjælpe med at lindre kløen. Derfor har mange studier fokuseret på interaktionen mellem kløe og smerte. For varmestimulationer har det vist sig, at langvarige smertefulde varmestimulationer kan hæmme smerte, men samtidig kan sådanne stimuli også forårsage hyperalgesi og hudskader.
Mange kliniske observationer peger på, at patienter kan opleve forøget kløe i varme miljøer, hvilket indikerer en forbindelse mellem varme og følelse af kløe.
Et 70 år gammelt studie viste, at der kunne opnås en paradoksal følelse af kløe, når bedøvet hud blev stimuleret med hvad, der blev defineret som en ”brændende” følelse.
Overordnet synes disse uafhængige observationer at indikere, at termisk perception kan spille en rolle i moduleringen af forskellige aspekter af kløe, og derfor er det interessant at opnå en bedre forståelse af de mekanismer, der ligger til grund for disse modulatoriske effekter.

Med baggrund heri var formålet med dette ph.d.-projekt at undersøge følgende aspekter: 1) Effekterne af meget korte varmestimuli (fra ikke-smertefulde til smertefulde) på forskellige kløe-modaliteter; 2)Hvordan kontinuerlig opvarmning af huden påvirker kløeintensiteten i humane surrugatmodeller for kløe; 3) Ved hjælp af en mere moderne og systematisk tilgang at undersøge og eventuelt bekræfte den observerede dysæstesi med paradoksal kløe, når der påføres en ”brændende” stimulation på bedøvet hud.
Det første studie viste, at homotopiske, kortvarige smertefulde varmestimulationer var i stand til at hæmme histaminerg kløe og i mindre grad ikke-histaminerg kløe fremkaldt ved hjælp af nåle fra planten mucuna pruriens. Når stimulationerne blev påført heterotopisk, var der ingen signifikant hæmning, hvilket antyder, at den observerede hæmmende effekt - i hvert fald for meget kortvarige stimuli – er afhængig af perifere mekanismer.

Det andet studie viste, at let opvarmning af huden selektivt forværrer serotonerg og histaminerg kløe men ikke kløe fremkaldt ved hælp af nåle fra mucuna pruriens. Disse resultater er til dels i overensstemmelse med observationer hos dyr (forøget serotonerg kløeintensitet) og andre dele er i direkte kontrast med disse (uændret histaminerg kløeintensitet).
Det tredje studie kunne ikke bekræfte det, som tidligere var en 70 år gammel observation. Påføring af en varmerampe (fra varm til smertefuld varme) på bedøvet hud fremkaldte ikke en paradoksal kløefølelse på nogen af de analyserede tidspunkter.
Denne afhandling præsenterer dermed varmestimulationers potentielle rolle til modulation af kløe. Yderligere studier er nødvendige for at undersøge denne rolle i klinisk kontekst.

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"All right," said the computer, and settled into silence again. The two men fidgeted. The tension was unbearable.
"You're really not going to like it," observed Deep Thought.
"Tell us!"
"All right," said Deep Thought. "The Answer to the Great Question..."
"Yes..!"
"Of Life, the Universe and Everything..." said Deep Thought.
"Yes...!"
"Is..." said Deep Thought, and paused.
"Yes...!"
"Is..."
"Yes...!!!...?"
"Forty-two," said Deep Thought, with infinite majesty and calm."

Douglas Adams

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CHAPTER 1. INTRODUCTION

1.1. ITCH AT A GLANCE

Itch is considered an uncomfortable sensation that causes the desire to scratch. It can be caused by different plants, animals or clinical conditions giving rise to acute or chronic itch[1–4].

Acute itch can occur in many instances with moderate to no effects on day-to-day life whereas chronic itch can be very burdensome.

Itch is primarily a skin condition, but the aetiology could originate from central neurobiological mechanisms or as secondary symptoms too eg. vascular conditions, kidney conditions, pharmacological treatments, etc…[5–7].

The desire to scratch is a primordial instinct and it is basically the urge to use mechanical pain to abolish the itch sensation. This consideration led the researchers to focus extensively on the interaction and the relationship between itch and pain transmission. When the pruritus sensation lasts for more than 6 weeks it is clinically referred to as chronic itch. To understand better the extent of such a condition, it has to be noticed that one every 5 people will experience chronic itch in their life[8,9]. In the most recent study on the global burden of disease (2017), “Skin and subcutaneous diseases” ranked ninth when measuring the “years lived with disability” parameter[10]. And among these diseases, the first four positions were held by atopic dermatitis, psoriasis, urticaria and scabies all of which are heavily characterized by the presence of itch[11]. It must be noticed that these results were before to the recent global pandemic that most likely would have skewed the results. Furthermore, chronic itch has been associated with a decrease in Health-Related Quality of Life (HRQoL) and can involve psychiatric comorbidities and sleep disorders [9,12–16].

Since 1990 there has been an increase in itch prevalence globally, from 812 to 932 in 2017 (out of 100000 people)[8]. Highlighting itch as a growing problem in our society. Interestingly, data indicates also being female and older as risk factors linked to itch[8,9,17–19].

The impact that itch diseases have on society can be also measured in health care expenditures. Only in the United States, itch has been associated with a yearly cost of more than $90 billion for the all population[8,20]. Altogether, these data highlight a clinical challenge that can affect a very large part of the population. Historically itch perception has been divided into a histaminergic and non-histaminergic itch, based on which receptors and pathways are involved.

1.2. SIMILARITIES AND DIFFERENCES BETWEEN ITCH AND PAIN

Traditionally, it is known that painful counter stimuli can inhibit itch. The same definition of itch recalls the desire to scratch which is substantially self-inflicted mechanical pain, that aims at removing the source of itch. Whereas the natural reflex for acute pain is a withdrawal response, intended to escape from the source that causes this sensation[21,22].

One of the main differences between these two sensory modalities is that pain can occur in various tissues (cutaneous, musculoskeletal, etc) whereas itch is present solely in the cutaneous tissue.

Interestingly, the known inhibitory effect of μ-opioids on pain does not affect itch sensation whereas μ-antagonists can inhibit itch and have no effects on pain sensation[23,24]. Whereas, κ-receptor's agonists showed to have analgesic and antipruritic effects, leading to the development of new potential drugs[25–27]. This line of evidence suggests a sort of specificity in the itch and pain transmission, in opposition to what has been widely demonstrated in the literature, where these two distinct sensory modalities have many receptors[28] and pathways[29–31] in common. Therefore, many theories have been postulated to evaluate and describe the relationship between these two sensory modalities.

1.2.1. ITCH CODING HYPOTHESES

Understanding how itch and pain are transmitted is important to identify new targets for a selective and effective therapy able to help the many patients suffering from chronic itch and chronic pain conditions.

Historically, itch has been considered a submodality of pain, giving rise to the so-called intensity theory according to which, some nociceptors can be activated by both algogens and pruritogens (hence the name polymodal fibres) and are connected to the same neurons' population at the central level. Depending on the intensity at which the neurons fire these can signal itch (weak activation) or pain (strong activation)[32–34].

The specificity theory (also referred to as labelled line theory) assumes that itch and pain are transmitted by fibres creating a distinct and selective pathway up to the central nervous system[32–34]. Many studies have identified various markers expressed in neurons attributed to specifically signal itch sensation[35–41].

The selectivity theory expands what is already said by the specificity theory by adding the presence of spinal interneurons activated by painful stimuli, that exerts an inhibitory effect on itch sensation[42].
The spatial contrast theory postulates that itch and pain sensation share the same set of neurons and nociceptors and the discrimination between these two sensory modalities is given by the number of nociceptors recruited in the epidermis. When there is a focal stimulation few nerve endings are activated, and this translate into an itchy sensation. When the area stimulated is larger, more nociceptors are excited, and this gives rise to a pain sensation[43,44].
Finally, more recently a leaky gate theory has been presented[45]. The latter has some similarities with the selectivity theory and shows a set of spinal neurons that are activated by both nociceptive and pruriceptive stimuli and can evoke simultaneously itch and pain sensations. Upon strong nociceptive stimulus, endogenous opioids inhibit what would be an overwhelming pain sensation.
Many studies in the last years have explored several aspects of itch and pain signalling and interaction, but to date, an unambiguous theory has not been presented. Further studies using both human and animal models are needed to understand how the brain and the nervous system can discriminate between itch and pain.

1.3. ITCH HUMAN MODELS

It is of utmost importance to study and understand itch mechanisms to develop new devices and therapies able to help to improve the patients' quality of life. To do so, many models have been developed both for animal and human experiments. Animal models could potentially provide a lot of information but unfortunately, they are not reliable when it comes to establishing the intensity of itch experienced, and they rely on behavioural responses such as scratching bouts and licking (itch and pain behaviour respectively)[46,47]. However, animal models remain the first choice when it comes to investigating new molecular mechanisms both at the central and peripheral levels. Several animal itch models have been developed and used in many studies, these will not be treated in detail here and further information can be found in literature[3,34,46,48].
A good way to study and investigate itch mechanisms is to use human surrogate models of itch. The current itch models, rely on the intracutaneous application of various substances, called pruritogens, producing a mild-to-moderate itch sensation. Except for cowhage-induced itch (where itch disappear as soon as the spicules are removed; more details below),not counter stimulated itch sensations will slowly fade when the substances in the

skin have been cleared physiologically. Although not optimal, these models helped in progressing toward a better understanding of the basic mechanisms of itch.

The most used pruritogen is histamine, able to evoke substantial itch, lasting between 5 to 20 minutes, coupled with a well-documented wheal and neurogenic inflammation (also referred to as neurogenic flare)[49–52]. The wheal appears as a small oedema (approximately 5 mm in diameter) in the exact provocation site resembling to the wheal present after a mosquitoes bite[49,52–56]. The neurogenic flare is characterised by a short increase in superficial blood perfusion due to the activation of peptidergic nerve fibers[57–59]. This latter cutaneous response, can be measured by full-field laser perfusion imaging (FLPI), a modern technique that can quantify the superficial blood perfusion by analysing the haemoglobin content[60–65].

Histamine activates histamine 1 receptor (H1) peripherally, inducing an increase in intracellular Ca^{2+} influx trough intracellular cooperation with phospholipase Cβ[66] or phospholipase A2[67]. Interestingly, it has been shown recently that selective and systematic inhibition of histamine 4 receptor (H4) lead to decreased itch intensity in rodents, highlighting a potential new role for H4[68]. Nonetheless, in clinic most chronic itch conditions are unaffected by the treatment with antihistamines, demonstrating how chronic itch does not rely solely on histaminergic pathways[1,69–72].

Histamine has to be introduced into the skin, to achieve an itch sensation. This can be done using a skin prick test, iontophoresis, intradermal injection and inactivated cowhage spicules soaked in a histamine solution[50,52,73–76].

Another itch human model is achieved by the use of cowhage spicules already characterized in 1955[77,78]. These are collected from the pod of *Mucuna pruriens* and applied onto the skin by gently rubbing with the fingertip until a light pricking sensation is felt on the treated skin. In recent years, cowhage received more attention as a pruritogen due to the unique sensory qualities that evokes[43,79]. The itch sensation evoked by cowhage is stronger compared to histamine, furthermore is coupled with nociceptive sensory qualities like pricking/stinging[43,80]. Cowhage-evoked itch lasts up to 5-15 minutes and rapidly disappears when the spicules are carefully removed[73,74]. The molecule believed to be responsible for its action is mucunain, an enzyme present on the spicules[78]. The mechanism that leads to the itch sensation is still not fully clear, two pathways have been suggested so far: the activation of proteinase-activated receptor 2 and 4 (PAR2/4) and it has been suggested the contribution of mas-related G protein-coupled receptor X1 and 2 (MRGPRX1/2)[35,81]. The activation of the MRGPRX2 also lead to mast cell degranulation, a mechanism that characterize histamine-dependent itch

pathways, suggesting that cowhage-induced itch pathways could also recruit histaminergic itch pathways[82–84]. Nonetheless, it has been shown that cowhage do not evoke any wheal typical of mast cells degranulation[50]. More molecular studies are needed to fully elucidate cowhage-evoked itch pathway. It is worth mentioning that all the receptors activated by mucunain are expressed and activated on superficial epidermal C-fibres[35,81].

A third model used for inducing itch in healthy subjects relies on the use of serotonin. Serotonin can be introduced to the skin by iontophoresis and induces a mild itch sensation[85,86]. A study explored itch induction by intradermal injection achieving slightly weaker results[87], overall iontophoresis remain the preferred induction modality. Serotonin in the epidermis activates both mechanosensitive, also called polymodal C-fibres (PmC-fibres), and mechano-insensitive C-fibres (Cmi-fibres), the latter a specific group that can be activated by histamine as well[88]. Notably, pre-treatment with antihistamines does not affect itch intensity following iontophoresis with serotonin[86], indicating that serotoninergic itch does not rely on histamine-releasing mechanisms to develop itch. However, treatment with tropisetron, a selective serotonin type 3 receptor (5-HT3) antagonist, showed a marked serotoninergic itch inhibition, suggesting that 5-HT3 may be the responsible for serotoninergic itch sensation[89].

Other itch models rely on the application of various pruritogens. Bovine adrenal medulla 8-22 (BAM8-22) can be applied to the skin to evoke an itch sensation similar to the one achieved with cowhage spicules[52,90–92]. BAM8-22 is a direct agonist of MRGPRX1 and can be introduced by the use of intradermal injection or inactivated cowhage spicules soaked in a solution of this compound[90].

The use of β-alanine as an agonist of MRGPRD has been used as itch model[93]. Qualitatively, it evokes a less intense itch compared to histamine- or cowhage-evoked itch. Furthermore, β-alanine is not able to provoke itch sensitization[94].

Transient Receptor Potential Ankyrin 1 (TRPA1) activation with trans-cinnamaldehyde lead to mild cutaneous pain as well as itch in some participants, but further investigation is needed to make it a reliable itch model.[95,96]. These latter three models were not used in the studies on which the dissertation is based, more detailed information can be found in the literature.

Thanks to these human models it is possible to analyse and investigate how itch interacts with other sensory modalities, as well as to develop new therapies.

For this thesis, the attention will be set on histamine-, cowhage- and serotonin-induced itch models.

1.4. HEAT PERCEPTION

Numerous studies have shown the effect of various thermal stimuli on top of the skin. These studies investigated the temperature at which generally warm detection and pain perception occurs.
Normal skin temperature is set around 32°C[95,97–100], and the Heat Pain Threshold (HPT) is ≈45°C[101]. These temperatures can vary based on different body regions stimulated, gender and method of stimulation[102–105].
Heat stimuli can be applied to the skin using various methods: radiation (infrared, microwave, laser, etc)[74,102,106], convection (through a fluid, i.e. air and liquid)[107,108] and conduction (by putting the skin in contact with a hot object)[73,97,109–111] or a combination of these. This dissertation will focus mainly on conductive heat transfer (using a controlled Peltier element; Study I and III) and radiant heat transfer (by the use of infrared radiation; Study II).
With regards to the interaction between itch and thermal stimulation, it has been shown how noxious heat and cold stimulation can inhibit itch sensation[73,97,110,112]. Specifically, these studies explored the effect of 30-seconds long stimulation at 49°C, showing that this was able to inhibit itch when applied homotopically and/or heterotopically to a previous itch provocation[97,109,110]. These results suggest that the inhibitory mechanism relied on spinal or supraspinal pathways, because the effect was observed also when the stimuli were applied heterotopically i.e. in a cutaneous area differently innervated compared to the itch provocation area[97]. Nonetheless, it is still unclear how this known mechanism can be exploited for the development of new therapies.

1.4.1. TRPs IN HEAT AND ITCH PERCEPTION

Heat perception resides mainly on a specific class of calcium-permeable ion channel receptors, of the TRPs family called Transient Receptor Potential Vanilloid (TRPV).
Two decades ago TRPV1 was indicated as the receptor able to sense noxious heat, and to respond to temperatures above 48°C; since then the research around these ion channels and their relation with thermal sensation has been widely explored[113,114]. Further studies showed that this receptor is activated by temperatures above 42°C[115,116]. Its central role as a primary noxious heat detector was confirmed also by genetic ablation experiments showing inhibition in heat pain sensation that was not completely abolished, suggesting

the involvement of other receptors as well[115,117,118]. TRPV1 is markedly expressed by small-diameter sensory neurons[113,117] as well as in keratinocytes[119,120].
Although direct activation of this receptor is known to cause pain and not itch[121], following itch induction with histamine, sensory neurons can intracellularly activate TRPV1[122]. Similarly, it has been shown how TRPV1 can help mediate itch when induced by PAR2 activation or IL-31[122,123].
TRPV2 activation starts at a higher temperature (>52°C), to date no studies are showing a connection between itch sensation and this receptor[115,124].
Warm innocuous stimuli activate TRPV3, specifically above 33°C[115,125]. This receptor is capsaicin-insensitive[125] and its expression is mainly in keratinocytes[126,127] rather than neuronal cells. Interestingly, a specific mutation of this receptor, characterized by a gain of function has been associated with pruritus and dermatitis[128,129]. Moreover, itch elicited by PAR2 activation has been linked with TRPV3[130,131].
Also, TRPV4 is activated by innocuous stimuli in the range of 27-42°C, the higher the temperature goes the more active this receptor is, and with repeated or sustained stimulation >42°C desensitization is achieved[115,132]. Furthermore, recently it has been shown its involvement both in serotoninergic and histaminergic itch[133–135].
Another TRP channel receptor is somehow involved in the heat perception, TRP Ankyrin 1 (TRPA1). TRPA1 is widely known for his role as chemosensor and in inflammation, but some studies also showed its involvement in thermal and mechanical sensation[136–139]. Although it has been recognised as a cold stimuli detector[115,140,141], it has been shown how its genetic deletion or pharmacological blockade is necessary in order to achieve a complete loss to noxious heat sensitivity[142]. One could think that these two sets of evidence contradict each other, nonetheless it has been shown that TRPA1 could in theory be able to exert this double sensitivity[143] and moreover some evidence in vitro showed activation with cold and heat stimuli[144]. Further investigations are needed to fully verify TRPA1 thermal sensitivity.

1.5. TOPICAL CUTANEOUS ANAESTHESIA

Local anaesthetic exerts their anaesthetic effect by blocking the sodium channels (Nav) and consequently blocking the majority of sensory fibres from firing any signals[145,146]. They have been extensively used in clinic for dermatologic procedures[147,148], in certain case they can also be used as treatments for particular neuropathic pain conditions in the form of plasters[149,150].

A recent study investigating the RNA expression profile of all the cutaneous sensory fibres showed that Nav channels are expressed by the so-called peptidergic (PEP) and non-peptidergic (NP) sensory fibres' endings in the epidermis[151]. According to this study, these fibres are involved in the pain and itch perception[151]. Therefore, topical Nav blockers are a potent tool to modulate the sensorial cutaneous inputs.
The use of topical anaesthesia, besides the well-known analgesic effect, has been reported to partly alleviate itch, usually in the form of anaesthetic creams[25,152,153]. These creams supposedly achieve anaesthesia in a large section of the skin, meaning that they can block fibres in the upper as well as the deeper cutaneous layers.
This distinction is important because it has been shown how the different fibres PEPs and NPs, involved in itch and pain transduction have their terminals ending in different cutaneous layers. Specifically, NPs terminals are in the upmost layer of the skin (superficial epidermis), whereas, PEPs endings are closer to the dermal-epidermal junction (deep epidermis)[151,154,155].
Interestingly, a 70 years old study investigated the effect of heat stimuli following topical anaesthetization due to subcutaneous injection of procaine (a local anaesthetic known to block the Nav channels)[156]. In this study, a stimulation intended to achieve a "decided sensation of burning" was applied on top of the anaesthetized skin and led to the development of a paradoxical itch sensation when anaesthetization started to subside[156]. This observation highlights a potential interaction between heat perception and itch pathways that needs to be further investigated to be fully understood.

1.6. AIMS OF THE PHD PROJECT

Given these premises, this PhD project aimed to explore and investigate how heat perception interacts with itch sensation and development. Furthermore, to understand if it can be used as a tool to modulate itch. For these purposes there were three main focuses:

1. To evaluate the effects of short noxious heat stimuli, homotopically and heterotopically to itch induction.
2. To investigate the pruritic effects of continuous warm innocuous stimulation pre- and post-itch induction in various human itch models.
3. To explore the short- and long-term effects of escalating heat stimulation on top of anaesthetised skin.
4.

1.6.1. PAPERS AND DISSERTATION OVERVIEW

To this end, the thesis is based on two published papers, and one submitted to peer-reviewed journals, each paper exploring one of the above bullet points.

- **Study I**[73]**: Riccio D.**, Andersen HH. & Arendt-Nielsen L. Antipruritic effects of transient heat stimulation on histaminergic and non-histaminergic itch. *Br. J. Dermatol.* **181**, 786–795 2019.
- **Study II**[74]**: Riccio D.**, Andersen HH. & Arendt-Nielsen L. Mild skin heating evokes warmth hyperknesis selectively for histaminergic and serotoninergic itch in humans. *Acta Derm. Venereol.* **102**, adv00649 2022.
- **Study III**[111]**: Riccio D.**, Lo Vecchio S., & Arendt-Nielsen L., The effect of escalating heat stimulation on top of anaesthetized skin. Itch 2022. (SUBMITTED).

All the studies were conducted on healthy human participants with the approval of the regional ethical committee (N-20180035). The full methodologies will not be discussed in this dissertation, a detailed version of them is available in the papers.
The figure 1 (Fig. 1) shows an overview of how the different studies analyse different aspects of the interaction between heat perception and itch.

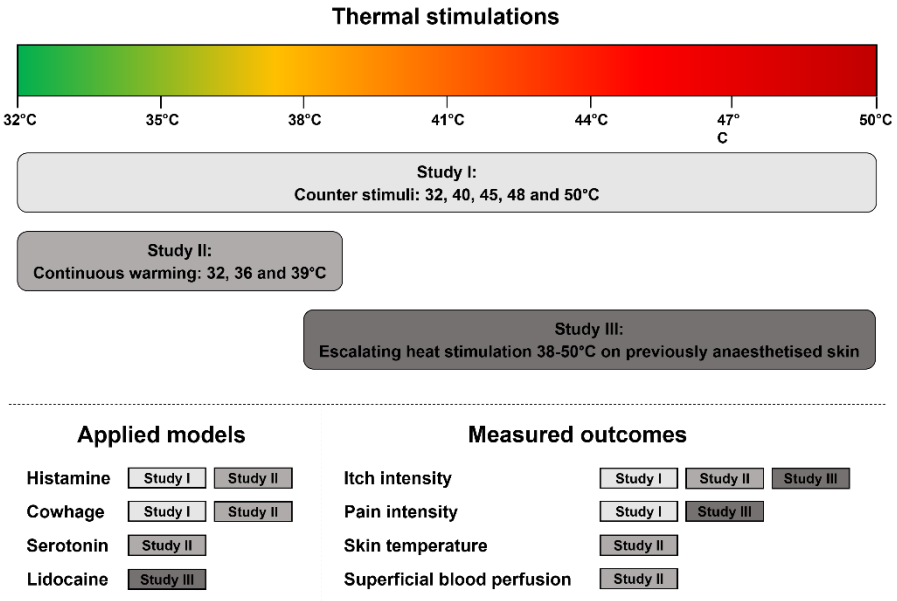

Figure 1. Schematic overview of the dissertation studies with the temperature ranges, models applied and outcomes measured: Study I and II investigated the effects of various heat stimuli on well-established itch models (histamine, cowhage and serotonin). Study III investigated the interaction between heat perception and anaesthesia (lidocaine).

CHAPTER 2. THE EFFECTS OF NOXIOUS HEAT ON ITCH

The content of this chapter is related to Study I[73].

In chapter 1.2 it was briefly discussed how counter stimuli can inhibit, partially or completely the itch sensation[5,157,158]. Particularly, it has been shown that long noxious heat stimulation can reverse itch in healthy human participants[97,109]. These long stimulations usually induce also primary and secondary hyperalgesia on the skin area stimulated and therefore they are not considered a suitable treatment option[159,160]. Nonetheless, it is important to investigate the mechanisms behind the observed effect as well as try a different approach to exploit this known outcome.
In Study I, the effects of very transient (5 seconds) heat stimuli in healthy participants after itch induction with histamine or cowhage has been analysed. The stimuli were applied, exactly 2 minutes after the itch provocation, at the same site as the itch induction (experiment 1, homotopically; Study I Fig.1A) or in a different site (experiment 2, heterotopically; Study I Fig. 1B) either next to the provocation site (intrasegmentally) or on the other side of the forearm (extrasegmentally; Study I Fig. 1C).
Itch intensity was measured starting from the itch induction up to 10 minutes after. Notably, the heat stimuli applied in Study I did not evoke a long-lasting pain sensation in any of the conditions, mainly due to their transient nature.

2.1 NOXIOUS HEAT REVERSE HISTAMINERGIC ITCH

Fifteen years ago Yosipovitch et al., started investigating the effects of noxious heat stimuli on histamine-evoked itch both in healthy participants[97,109] and atopic dermatitis patients[110]. As mentioned before, these focused on long noxious stimuli (30 seconds stimulations), applied homotopically or heterotopically. Little is known about the effects of transient and very short heat stimuli.
The results in Study I show that a very transient homotopical noxious heat stimulation was able to greatly inhibit histaminergic itch sensation. Specifically, a significant reduction in itch sensation was achieved with stimulations of 45°C (-41.27%, $p<0.01$) and 50°C (-76.66%, $p<0.0001$; Study I Fig. 2A)[73].

Interestingly, as explained in section 1.4, these stimuli, able to inhibit itch, directly activate TRPV1[115,161], known to hold an important role in signalling histaminergic itch[162–166]. Following histamine receptor activation, intracellular pathways lead to the co-activation of TRPV1[91,167].
It can be speculated that, due to the external activation of TRPV1 with transient heat counter stimulation, histaminergic intracellular pathways are not able to co-opt it and therefore an inhibition in itch sensation is observed. This latter hypothesis is yet to be confirmed, with further investigations and possibly with pharmacological tools.

2.2 NOXIOUS HEAT PARTIALLY INHIBITS NON-HISTAMINERGIC ITCH

With regards to cowhage-induced itch, Study I shows that a homotopic transient noxious stimulation (50°C) was able to achieve a partial itch inhibition (-43.6%, p=0.011; Study I Fig. 2B).
As explained in section 1.3, cowhage spicules lead to the activation of PAR2/4 and/or MRGPX1/2[81]. Rather than recruiting TRPV1 as for histaminergic itch, these pathways are known to recruit TRPA1[168,169], which is substantially less activated by heat noxious stilmuli[142,170,171]. As mentioned in the section 1.4, TRPA1 historically has been considered as a cold detector but some evidence showed a possible activation by heat stimuli, that needs further data to be confirmed[144].
Since cowhage-induced itch was inhibited to a lower extent (compared to histaminergic itch; Study I Fig. 2B and Fig. 3) and there was no significant inhibition by stimuli lower than 50°C, one can assume that the different inhibition pattern derives from the different TRP receptors involved, specifically cowhage-induced itch was less inhibited due to the less prominent response to heat stimuli by TRPA1[73].

2.3 HETEROTOPIC NOXIOUS HEAT DOES NOT AFFECT ITCH SENSATION

The second experiment conducted in Study I, explored the effect of heterotopic counter stimuli to itch induction.
For both histaminergic and non-histaminergic itch, heterotopic stimuli were not able to inhibit itch sensation (Study I Fig.5).
The effect known as “diffuse noxious inhibitory control” was for the first time described in rodents in 1979[172,173]. This psychophysical mechanism refers to the reduced pain perception following a painful stimulus applied to a

heterotopic area. Later on, the human behavioural correlate was investigated and named conditioned pain modulation (CPM)[174,175]. Studies have shown that CPM signal starts at central level in the brainstem[175–178]. Thus, heterotopic counter-stimuli to the itch induction site were explored to rule out the presence of a similar mechanism called conditioned itch modulation[179,180].

As showed in Study I, both histaminergic and non-histaminergic itch were inhibited only by homotopical counter-stimuli (Study I Fig. 5). This is in sharp contrast with pieces of evidence showing pain-evoked itch inhibition being mainly a central mechanism because itch inhibition by intra- and extrasegmental counterstimuli would have meant the central nervous system involvement[42,181].

Furthermore, some previous studies presented opposite results to what showed in Study I. Specifically, itch inhibition was achieved by intrasegmental stimuli in the noxious range[97,109,110,182]. It has to be notice that in all of these studies the counter stimulations were long (3 minutes[182] and 30 seconds[97,109]) in opposition to the present study in which the stimuli lasted 5 seconds to avoid any possibility of hyperalgesia (both primary and secondary). The transient and short nature of the applied stimuli could be the reason for lack of involvement of the descending inhibitory pathways on the itch sensation.

Taken together the results of this study show that depending on the type and the pathways involved, itch can be differently modulated by counter-stimuli.

CHAPTER 3. THE EFFECTS OF INNOCUOUS HEAT ON ITCH

The considerations made in this chapter are based on Study II[74].

Itch is defined as chronic when it persists for more than 6 weeks and generally comes with two characteristic symptoms: alloknesis (when a non-pruritogen stimulus causes itch sensation) and hyperknesis (when a pruritogen stimulus causes a stronger and/or longer itch sensation)[183–185]. Many studies have focused on a specific type of dysesthesia called warmth hyperknesis, the mechanism where skin warming lead to an exacerbation in itch intensity, both in animal models[108] and in patients[186–188]. Nonetheless, a clear understanding of the mechanism characterizing warmth hyperknesis is lacking and therefore further investigations are needed.
Study II aimed to explore the effect of mild skin warming on various human itch models. To do so, the skin of the volar forearms of the healthy subject was heated either by +4 or +7°C above their measured and recorded individual baseline (≈32°C) using an infrared lamp. Once the target temperature was achieved, itch was provoked either by histamine, serotonin or cowhage, and itch intensity was assessed for up to 10 minutes, with constant and precise skin warming by the infrared lamp (Study II Fig1).
Furthermore, it was also investigated the superficial blood perfusion at two different time points after the skin warming and the itch intensity recording were stopped (Study II Fig1).

3.1 SELECTIVE WARMTH HYPERKNESIS FOR HISTAMINERGIC AND SEROTONINERGIC ITCH

Histaminergic itch intensity was significantly increased when the skin was warmed by 7°C (≈39°C), the same effect was not observed when the skin temperature was increased by 4°C (≈36°C; Study II Fig.3). This result is in direct contrast with what was previously reported in animals, where histaminergic itch was not enhanced at any of the analysed temperatures[108]. Interestingly, the warmth hyperknesis phenomenon was present only in the first 2 minutes after itch induction, meaning the onset phase, and not at later stages.
Serotonin-evoked itch intensity was enhanced when the skin temperature was increased by 4°C (≈36°C) and not by 7°C (≈39°C; Study II Fig. 4). Animal preclinical findings confirm these data, where serotoninergic itch was

enhanced when the skin temperature was ≈36°C[108]. Also, in this case, the increased itch sensation was present in the early part after the itch provocation, specifically in the first 4 minutes (Study II Fig.4).
As explained in section 1.4.1 of this dissertation, TRPVs have a major role in transmitting heat perception from innocuous to noxious[115]. The temperatures used in Study II are mainly sensed by TRPV3 and TRPV4 (≥33°C and 27-42°C respectively). TRPV4 has been recently linked to histaminergic itch[133] and serotoninergic itch[134,135], whereas a direct interaction between TRPV3 and these itch modalities has yet to be shown. Therefore, it can be speculated that there is the direct involvement of TRPV4 in the hyperknesis effect hereby observed, but other key players cannot be ruled out.
Of particular interest is the observation that hyperknesis occurred only in the initial phase of the itch sensation (Study II Fig.3-4), suggesting that the itch modulation by activation of TRPVs is more relevant in the onset phase and at later stages is less important (in regard to the itch intensity).

3.2 COWHAGE-INDUCED ITCH IS NOT AFFECTED BY INNOCUOUS SKIN HEATING

Cowhage-evoked itch intensity was not modified by the increase in skin temperature both by 4 and 7°C (Study II Fig.5). Interestingly, even though these results showed a lack of itch modulation following TRPV3 activation (via temperature), it has been shown how TRPV3 is important in the itch transmission when evoked by cowhage[130]. Specifically, when keratinocytes lacked TRPV3, there was a decreased itch behaviour in mice, when treated with PAR2 agonists (compared with wild-type animals)[130]. One could assume that, despite the well-documented interaction, prior activation of TRPV3 is not able to exacerbate itch intensity. It could be that cowhage spicules activate PAR2 which in turn recruits TRPV3 to exert its pruritic effect, but further or greater TRPV3 activation is unable to increase itch intensity.
Another reason for the lack of warmth hyperknesis in this condition could be that pre-activation of TRPV3 does not facilitate itch.
But we cannot exclude the possibility of increased warmth sensation given that this was not recorded in the experiment.

3.3 ENHANCED SUPERFICIAL BLOOD PERFUSION DUE TO INNOCUOUS SKIN HEATING

Other than itch intensity, Study II explored the combined effect of different individual itch provocation and mild skin warming onto superficial blood

perfusion. The results from this analysis showed that superficial blood perfusion following histaminergic itch induction was unaffected by the continuous increase in skin temperature (Study II Fig. 6). It is known that histamine-evoked itch alone can generate a substantial neurogenic inflammation with subsequent increased blood perfusion together with cutaneous redness and wheal[51]. Therefore, one could suggest that higher skin temperature does not increase this state any further.

Superficial blood perfusion was significantly enhanced when skin temperature was increased by 7°C for serotoninergic itch, while for cowhage-evoked itch it was increased both at +4 and +7°C above the resting skin temperature. This line of evidence confirm, what previously observed, that neurogenic inflammation is not necessary for the development of itch sensation because the first was increased exactly when the latter was not different from the control condition[49,80,92,189,190]. A very captivating explanation could be that increased superficial blood perfusion leads to increased tissue clearance and therefore this would counteract what would be an increased itch intensity, but this study does not provide enough data to make such an assumption. Further investigations in this regard are needed.

It has been shown that TRPV3 and PAR2 participate in the inflammatory response[127,191–195]. Therefore, the increased blood perfusion could be due to the probable co-activation of these two receptors that would lead to a more pronounced inflammatory response.

Regarding serotonin-evoked itch, increased blood perfusion was achieved only when the skin was heated by 7°C. As we said earlier, to date there is no evidence suggesting an interaction between serotoninergic itch and TRPV3, rather with TRPV4[134,135]. Temperature-selective increased neurogenic inflammation in this setup could be due to a marked activation of TRPV4 (i.e. skin temperature ≈39°C); once again this is just a speculation that needs to be confirmed.

CHAPTER 4. THE EFFECTS OF ESCALATING HEAT ON TOP OF ANAESTHETIZED SKIN

This chapter is based on the results shown in the manuscript submitted, Study III[111].

As discussed throughout the introduction and the previous studies of this dissertation, the relationship between heat and itch is complicated and has many aspects to be analysed. In section 1.5 it was mentioned how topical cutaneous anesthetisation is able to inhibit both pain and itch by blocking Nav channel transmission[25,145,146,152,153,196]. There is some evidence regarding the effectiveness of topical anaesthetics in various itch conditions, particularly neuropathic itch[25,152,197,198]. However, the use of these topical anaesthetics is not a feasible clinical solution to itch and pain, especially when these are chronic.

Study III investigated whether escalating heat on top of anaesthetized skin can provoke a paradoxical itch sensation when the anaesthesia is subsiding. This was done to explore, using a more modern and systematic approach, the results achieved by a 70 years old study[156]. In this study, the skin was anaesthetised by 2% procaine with subcutaneous injections. Once the efficacy of the anaesthesia was verified by pinprick sensibility, the area was heated by an infrared lamp to 41°C. After 15 minutes from the anaesthetisation, a paradoxical itch sensation started to develop, while simultaneously numbness was still present but pinprick sensibility was recovered[156]. To date, we are not aware of any further study investigating this peculiar mechanism, that could be useful to develop new sensitive targets.

In Study III, healthy skin was superficially anaesthetized by using intradermal injection with lidocaine 2% together with adrenalin as vasoconstrictor to achieve a qualitative better anaesthetisation[199]. Subsequently, on top of the same spot, an escalating heat ramp, lasting 1 minute in total and ranging from 38 to 50 °C, was applied every five minutes. To the participants it was asked to continuously rate their itch and pain sensation simultaneously during every heat stimulation (Study III Fig.S1). The results were compared with saline treatment as internal control.

To have a better idea of the different modulations involved in this study, the analysis of the itch and pain sensation was divided into 3 subgroups according to the temperature at which the intensity was rated. Innocuous range i.e. 38-42°C, painful range i.e. 42.2-46°C and finally noxious range i.e. 46.2-50°C.

4.1 PAIN INTENSITY

Pain sensation was reduced in the area treated with lidocaine compared to saline, specifically in the noxious range in the first 15 minutes (Study III Fig.1). This result suggests that, after this period, the anaesthetisation is subsided enough to not be able to inhibit strong nociceptive stimulation.
Interestingly, a previous study showed that an intradermal injection of lidocaine led to anaesthetisation being present after 20 minutes and subsiding after 120 minutes, this was tested by various heat stimuli ranging from 40 to 52°C[200]. Whereas, Cormia and Kuykendall[156] started to see the aforementioned "paradoxical itch sensation" exactly 15 minutes after the anaesthetic treatment.
When the escalating stimulation was in the painful and noxious range, 40 minutes after the anaesthetisation, it was observed a decreased pain intensity. To the best of our knowledge, this pain inhibition at this specific time point does not have a clear explanation and further studies are needed to assess whether it was an artefact or a specific unexplored new mechanism.

4.2 ITCH INTENSITY

The results in Study III showed no significant differences between saline and lidocaine treatment in itch intensity, in any of the temperature ranges and time points analysed. This is in sharp contrast with what was observed 70 years ago[156].
As explained in section 1.5, a recent study characterized the different sensory fibres by discriminating them according to their transcriptome[151]. The leading hypothesis prior to Study III was that after about 15 minutes (as previously showed[156]) the fibres of the uppermost layer (superficial epidermis[154,155]) (i.e. NP fibres) would recover faster from the anaesthetisation and therefore would transmit a paradoxical itch sensation, as a consequence to noxious heat stimulation. For this reason, intradermal injections were preferred to a more common and modern method such as anaesthetic cream (e.g. EMLA), which would achieve a deeper, longer and less specific anaesthetisation.
It has to be pointed out that in the previous study, the temperature at which it was achieved an itch sensation was constant and developed by a radiant heat source, in opposition to Study III in which there was a heat ramp by direct contact with the skin. This methodological difference could have been what led to the development of paradoxical itch sensation due to the prolonged stimulation. Further studies are needed to explore different heat modalities and more specific temperatures in this setup to understand better the mechanisms at play.

CHAPTER 5. GENERAL DISCUSSION

There is a need for new targets and therapies able to help chronic itch patients. The three studies presented in this thesis explore how heat from innocuous to noxious range can modulate itch. The ultimate goal of these studies was to understand which receptors and/or pathways involved in the processing of thermal sensation can be exploited toward the development of new treatments.
In brief the take home messages are:
-1) Short noxious heat stimuli can greatly inhibit itch.
-2) Innocuous skin warming can exacerbate itch.
-3) Heat stimuli onto anaesthetized skin are not able to evoke itch sensation.

1) Very transient (5 seconds) noxious homotopical heat stimuli can inhibit different modalities of itch (histaminergic and cowhage-induced itch) to a different extent. Specifically, histaminergic itch showed a greater inhibition compared to cowhage-evoked itch (up to 76% compared to 43%, respectively[73]).
Recently, some tentative device has been developed and studied in a clinical context, consisting of the application of 51°C for 3 or 6 seconds on top of insect bites[201]. This latter study, showed how swelling, pain intensity and itch intensity, coming from wasps, bees or mosquitoes bites, were greatly inhibited, demonstrating that transient noxious heat stimuli can help in modulating itch intensity[201].
Although, it is widely known that itch sensation due to insect bites is primarily histaminergic and this study can provide some knowledge about the intricate interaction between itch and pain, further systematic investigations are needed to better understand the mechanisms at play.
It has to be kept in mind that this approach would be scarcely applicable in chronic itch patients due to the large section of skin on which noxious heat should be applied as well as probably repeated exposure during the day.
Moreover, most of the itch symptomatic conditions (except for urticaria and mastocytosis) are unaffected by the treatment with antihistamines[25,152] therefore it is important to find mechanisms able to inhibit mostly non-histaminergic itch.
For this reason, Study I focused more on the potential mechanism underlining such an effect and the involvement of TRPV1 (important for the development of histaminergic itch[91,162–167]) and TRPA1 (recruited during cowhage-induced itch[168,169]) was hypothesized.

Capsaicin (a potent TRPV1 agonist) is used in clinical setup as an 8% cream able to achieve on some occasions long-lasting itch relief[153,202–206]. Whereas, to the best of our knowledge compounds acting on TRPA1 are yet to be explored as potential drugs for treating itch.
The data presented in Study I together with these clinical observations strongly suggest continuing the research toward TRPV1 and/or TRPA1 activation as a valuable targets to inhibit chronic itch.

2) The known mechanism of warmth hyperknesis[108,186–188], was observed when itch was evoked by histamine and serotonin and not by cowhage. It can be assumed that the enhanced itch sensation could originate from the activation of TRPV4 rather than TRPV3, because of the selective documented interaction that this receptor has with both histaminergic[133] and serotoninergic itch[134,135] pathways. Moreover, despite the known interaction between cowhage-induced itch and TRPV3[130], the pre-activation by skin warming of this latter receptor did not result in enhanced itch intensity.
TRPV4 is generally expressed by both sensory neurons and keratinocytes[207]. In keratinocytes, other than participate in itch development of histaminergic and serotoninergic itch, TRPV4 activation leads to the release of endothelin 1, known for its pruritogen activity[67,133,208]. Interestingly, this evidence shows also that keratinocytes are able to initiate itch sensation without the involvement of neuronal cells[34].
Given these premises, a potential new target for therapies could be the antagonism of TRPV4. To the best of our knowledge, such a mechanism has yet to be explored for clinical purposes.

3) By using a more systematic and modern approach to investigate a previously observed effect[156] data presented in Study III did not confirm the results from an old study, however, few considerations can be done.
Firstly, if the Study III hypothesis would have been confirmed, could have given a new potential target by retro-analysing at what exact temperature itch sensation occur and therefore which among the thermal receptor was most likely involved in this mechanism.
Secondly, in the paper published by Cormia and Kuykendall, the stimulation able to evoke a paradoxical itch sensation was achieved by irradiation with an infrared lamp. The same methodology was used in Study II to mildly warm the skin which resulted in hyperknesis. Together these pieces of evidence could suggest that radiant heat stimulation, specifically using an infrared lamp, can facilitate itch sensation in certain conditions. This would be a novel and

unexplored role for such a stimulation but need further investigations to be verified and understood.

All things considered, the results and speculations highlighted in this dissertation point out that heat perception and TRP receptors as key player in itch sensation, that need more attention in the development of new potential therapies.

5.1 LIMITATIONS

The studies hereby analysed present some limitations.
In Study I[73], only cowhage was used as a non-histaminergic itch model. Whereas, in Study II it was used also serotonin-evoked itch. This was done for practical reasons, serotoninergic itch is induced by iontophoresis which requires more space on the skin due to the presence of the conductive pad and ion chamber (compared to histamine- and cowhage evoked itch, which requires only the treated area). Therefore having 5 different areas on the forearm (as for histamine- and cowhage-evoked itch) would have been not feasible. Moreover, the second experiment of Study I would have been impossible to be performed in a single session without applying itch provocation on the same area already treated previously (Study I Fig.1B-C). We could hypothesize that serotoninergic itch would have been inhibited by noxious heat as it happened for the other 2 itch modalities, to understand to what extent further investigations are needed.
The analysis of the skin blood perfusion in Study II[74] was not adopted in the other two studies. This was due to the intensity of the heat stimulation applied that most likely would have resulted in an oversaturated picture, to be expected due to the cutaneous redness seen after the heat stimulations (data not shown).
The heat stimulations applied in the three studies differ substantially from the qualitative point of view. In Study I and III contact heat stimuli were used, whereas in Study II it was used a source of radiant heat. The latter was chosen to better simulate what was observed in clinic, where patients have exacerbated itch in warm environments. Moreover, by applying a contact form of stimulation the results could have been invalidated due to mechanical itch inhibition.
In Study I and II more intermediate temperatures could have been explored, however, due to limited space on the forearms (Study I Fig.1 and Study II Fig.1) this was not possible.
On the other hand, in Study III it could have been more helpful to use very specific heat stimuli (i.e. few chosen temperatures) instead of using a heat

ramp. However, our purpose was to understand at exactly what temperature the potential paradoxical itch sensation would occur, to have a systematic view of the effects at play, for this reason, a heat ramp was preferred.

CHAPTER 6. CONCLUSIONS

This dissertation aimed to evaluate 1) the effects of transient noxious heat stimuli on itch, 2) investigate how various itch models interact with continuous warm innocuous heat stimulation and 3) explore old results showing a "paradoxical itch sensation" when anaesthetised skin receives a "burning" heat stimulation[156].

Study I[73] showed that transient homotopical noxious heat counter stimulations can inhibit itch showing a dose-response effect, where the higher the temperature the higher the inhibitory effect is. These are more effective on histaminergic compared with non-histaminergic itch. Moreover, heterotopic counter stimuli failed to inhibit itch, suggesting that transient heat stimuli effect is mainly due to peripheral mechanisms.

Study II[74] partially confirmed what was already observed in animal[108] where serotoninergic itch was enhanced by innocuous skin warming. In contrast with data in rodents, it also showed exacerbation of histaminergic itch due to skin warming. These data may give a more detailed explanation of what has been observed in clinic[186–188], the so-called warmth hyperknesis and open up a new avenue in the development of new therapies by antagonising TRPV4, the main suspect in the observed itch exacerbation.

Interestingly, cowhage-induced itch was not aggravated, however, there was an increased skin blood perfusion. This was probably due to the co-activation of PAR2 (by cowhage spicules) and TRPV3 (by increased temperature) known to interact[130] and probably cause an increased inflammatory response.

Study III failed to confirm what was observed in an old study[156], since noxious heat applied on top of previously anaesthetised skin did not evoke any paradoxical itch sensation. As briefly explained in section 5.1, it could be useful to investigate the effect of specifically chosen temperature to better match the methodology used in the previous study.

In summary, these studies highlight a unique role in the interaction between heat and itch perception and intensity, suggesting how fine-tuning and modulation of TRPVs receptors can translate into inhibition or increased itch intensity. Particularly, following up on the use and the development of TRPV1 agonists and exploring the effect of endogenous and synthetic TRPV4 antagonists.

Further proof-of-concept studies in patient populations are needed to validate the experimental data and the initial step toward a better understanding of new non-pharmacological targets to treat chronic itch.

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